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ORIGINAL ARTICLE

Response surface methodology study of the combined effects of temperature, pH, and a_w on the growth rate of *Trichoderma asperellum*

B.A.D. Begoude[1,2], R. Lahlali[2], D. Friel[2], P.R. Tondje[1] and M.H. Jijakli[2]

1 Laboratoire Régional de Lutte Biologique et de Microbiologie Appliquée, Institut de le Recherche Agricole pour le Développement (IRAD), Nkolbisson, BP 2067, Yaoundé, Cameroun

2 Unité de Phytopathologie, Faculté Universitaire des Sciences Agronomiques de Gembloux, Passage des Déportés 2, Gembloux, Belgique

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modelling, pH, radial growth rate, temperature, *Trichoderma asperellum*, water activity.

Correspondence
M.H. Jijakli, Plant Pathology Unit, Gembloux Agricultural University, Passage des Déportés 2, 5030 Gembloux, Belgium.
E-mail: jijakli.h@fsagx.ac.be

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Abstract

Aims: To evaluate the influence of environmental parameters (water activity a_w, temperature, and pH) on the radial growth rate of *Trichoderma asperellum* (strains PR10, PR11, PR12, and 659-7), an antagonist of *Phytophthora megakarya*, the causal agent of cocoa black pod disease.

Methods and Results: The radial growth of four strains of *T. asperellum* was monitored for 30 days on modified PDA medium. Six levels of a_w (0·995, 0·980, 0·960, 0·930, 0·910, and 0·880) were combined with three values of pH (4·5, 6·5, and 8·5) and three incubation temperatures (20, 25, and 30°C). Whatever the strain, mycelial growth rate was optimal at a_w between 0·995 and 0·980, independently of the temperature and pH. Each strain appeared to be very sensitive to a_w reduction. In addition, all four strains were able to grow at all temperatures and pH values (4·5–8·5) tested, highest growth rate being observed at 30°C and at pH 4·5–6·5. The use of response surface methodology to model the combined effects of a_w, temperature, and pH on the radial growth rate of the *T. asperellum* strains confirmed the observed results. In our model, growth of the *T. asperellum* strains showed a greater dependence on a_w than on temperature or pH under *in vitro* conditions.

Conclusions: a_w is a crucial environmental factor. Low a_w can prevent growth of *T. asperellum* strains under some conditions. The observed and predicted radial growth rate of strain PR11 showed its greater capacity to support low a_w (0·93) as compared with other tested strains at 20°C. This is in agreement with its better protective level when applied in medium-scale trials on cocoa plantations.

Significance and Impact of the Study: This study should contribute towards improving the biocontrol efficacy of *T. asperellum* strains used against *P. megakarya*. Integrated into a broader study of the impact of environmental factors on the biocontrol agent–pathogen system, this work should help to build a more rational control strategy, possibly involving the use of a compatible adjuvant protecting *T. asperellum* against desiccation.

Introduction

The cocoa tree (*Theobroma cacao* L.) is cultivated for its economically important bean. It is among the most developed cash crops in Western and Central Africa, and its production represents 70% of the 3 million tons produced worldwide (ICCO, International Cocoa Organisation 2003).

The global rise in cocoa production is limited by many constraints, such as cocoa black pod disease. Different species of *Phytophthora* are known to cause this disease, and these species vary according to both their aggressiveness and the level of crop loss caused (Appiah *et al.* 2004). *Phytophthora megakarya*, which prevails in Cameroon, is the most aggressive of the four main species of *Phytophthora* causing black pod disease of cocoa, with crop losses ranging from 60 to 100% (Ndoumbè-Nkeng *et al.* 2004). *Phytophthora palmivora*, regarded as the most widespread cocoa pathogen, can cause crop losses of 20–30% (Iwaro *et al.* 1997). It is more aggressive than *Phytophthora capsici*, the major *Phytophthora* species in Brazil (Keane 1992). *Phytophthora citrophthora* is the least widespread *Phytophthora* species, present only in the cocoa-growing region of Brazil. It was reported to be more aggressive than *P. palmivora* and *P. capsici* (Lawrence 1998).

Classically, cocoa growers use farming strategies, genetic strategies, and most often chemical strategies to minimize the impact of black pod disease (Akrofi *et al.* 2003; Efombagn *et al.* 2004; Ndoumbè-Nkeng *et al.* 2004). The main chemicals used are copper- and metalaxyl-based fungicides (Matthews *et al.* 2003). Beside the high cost of chemical treatments, a heavy reliance on them can be associated with nontarget effects, loss of biodiversity, spoilage of land and water, and the development of pathogen resistance (He *et al.* 2005).

Biological control by means of antagonistic microorganisms is an emerging strategy in many countries affected by this disease (Krauss and Soberanis 2001). In Cameroon, research focusing on biological control of *P. megakarya* began in 1999. It has led to the isolation and identification of four mycoparasitic strains of *Trichoderma asperellum* (PR10, PR11, PR12, and 659-7) (Tondje *et al.* 2003). Large-scale trials carried out with those strains, following an aqueous conidial preparation revealed inconsistent results.

The variable performance of *T. asperellum* as a biocontrol agent could be because of the influence of environmental factors that vary in time and from farm-to-farm. According to Magan and Lacey (1988) and Plaza *et al.* (2003), water availability (a_w) and temperature are the principal abiotic parameters determining the germination and growth potential of micro-organism propagules. No literature is available concerning the effect of these environmental parameters on the development of *T. asperellum*.

To optimize the practical use of a biological control agent, it is essential to understand how the physical environment affects the agent's survival, growth, and reproduction (Fravel 1999; Sanogo *et al.* 2002). Response surface methodology (RSM) is the approach most often used to describe relationships between a combination of factors and an organism's growth curve parameters (Devlieghere *et al.* 1998). As no mathematical model for *T. asperellum* is described in the current scientific literature, the aim of the present work was to elaborate models predicting the combined effects of temperature, pH, and a_w on the growth of *T. asperellum* strains (PR10, PR11, PR12, 659-7) under controlled conditions.

Materials and methods

Micro-organisms

Four strains of *T. asperellum* (Table 1) were obtained from mixed farmer's fields near Yaounde, Cameroon. Three (PR10, PR11, PR12) were isolated from roots and soil surrounding *Xanthosoma sagittifolium* and one (659-7) was isolated from soil under banana. They are conserved in collections at the Regional Biological Control and Applied Microbiology Laboratory of IRAD (Cameroon) and the Centraalbureau voor Schimmelcultures (CBS). The cultures were first identified microscopically as *T. asperellum*; microscopic identification was confirmed through sequences of translation-elongation factor 1-alpha (*tef1*) and sequences were deposited in GenBank (Table 1). A portion of *tef1* was amplified using the primers EF1-728F (Carbone and Kohn 1999) and TEF1 rev (Samuels *et al.* 2000). The PCR product of approximately 600 bp covered the large fourth and the short fifth introns of the gene. For the long-term storage, the strains were placed in tubes containing 25% (v/v) of glycerol and incubated at –70°C. In the experiments, the initial inoculum was taken from culture on potato dextrose agar (PDA) medium in petri dishes preserved at 4°C for not more than 6 months.

Medium

The basic medium used was PDA (a_w = 0·995). The a_w was modified by adding increasing amounts of glycerol to obtain a_w levels of 0·980, 0·960, 0·930, 0·910, and 0·880 at three different temperatures (Lahlali *et al.* 2005). The medium was buffered with 0·1 mmol l^{-1} NaH_2PO_4, the

Table 1 Strains of *Trichoderma asperellum* used

	GenBank number
Strain	*tef*
PR 10	EF185998
PR 11	EF186002
PR 12	EF185999
659-7	EF186001

final pH of the medium being adjusted to 4·5 with 80% H_3PO_4 or to 6·5 or 8·5 with 1 mol l^{-1} NaOH before autoclaving. The a_w of all the media was measured with an AquaLab series 3 instrument (Decagon, 950 NE Nelson Court Pullman, Washington 99163).

Preparation of the inoculum

A 10-day-old colony culture of *T. asperellum* grown on PDA was used to obtain spore suspensions. Ten to twenty millilitres of sterile distilled water containing 0·05% Tween-20 was added to the petri dish and conidia were carefully scraped from the surface of the colonies before filtration through sterile cheese cloth. Spore suspensions were adjusted to 10^6 spores ml^{-1} with a Bürker cell, then 10-µl aliquots of suspension were inoculated at the centre of the petri dishes containing the test medium. After inoculation, the petri plates were sealed in polyethylene bags to prevent water loss and then incubated for 30 days at 20, 25, or 30°C. Preservation of the water content of the media was checked by measuring the a_w in inoculated petri dishes after 25 days at each temperature. No change in a_w was detected in any medium (Lahlali *et al.* 2006).

Data recording

The radius of each growing mycelial colony was measured daily in two perpendicular directions (Marín *et al.* 1996), without opening the petri dishes, until the plates were completely colonized. Four replicates were used for each combination of experimental conditions. The radial growth rate (mm day^{-1}) for each a_w, temperature, and pH combination was obtained from linear regression slopes of the temporal growth curves (Lahlali *et al.* 2005).

Statistical analysis

Growth rates were subjected to the general linear model procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Statistical significance was done at the $P < 0·05$ level. Where analysis revealed significant differences, Duncan's multiple-range test for separation of means was performed.

Experimental design

RSM with a 3^k factorial design was applied with the STATGRAPHICS Plus version 3 statistical software. Temperature (20, 25, and 30°C), a_w (0·980, 0·930, and 0·880) and pH (4·5, 6·5, and 8·5) were investigated (Table 2). Data modelling was performed by multiple regression analysis. The design contained 27 experiments with four replicates. A second-order polynomial model was defined to fit the response:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1} \beta_{ij} X_i X_j$$

where Y is the response (growth rate), β_0 is a constant coefficient, X_i are coded variables ranging from –1 to +1, β_i represent linear coefficients, β_{ij} are the second-order interaction coefficients, and β_{ii} are the quadratic coefficients. All values of model coefficients were calculated by multiple regression analysis. Interpretation of the data was based on the signs (positive or negative effect on the response) and statistical significance of coefficients ($P < 0·05$). Interactions between two factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient). The R^2 value (coefficient of determination) expressed the variation of Y explained by the model of regression (three variables studied, X_i).

Results

Effect of a_w, temperature, and pH on the growth rate of *Trichoderma asperellum* strains

The growth lag times and radial growth rates (mm day^{-1}) of *T. asperellum* strains PR10, PR11, PR12, and 659-7 were determined for each combination of temperature, a_w, and pH. The results are shown in Figs 1 and 2 and in the 'observed values' columns of Table 2.

At 25–30°C and at any pH, the growth lag was about the same (1 day) for all strains at a_w down to 0·960. At 20°C, strains PR10, PR11, and PR12 showed an increased growth lag (2 days or more) from $a_w = 0·960$ downward.

At all temperatures and at any pH, all strains showed a growth rate decreasing with decreasing a_w. The fastest growth observed at $a_w = 0·980$ was 14·80 ± 0·09 mm day^{-1} (strain PR12, 30°C, pH 4·5). In contrast, the highest growth rate observed at $a_w = 0·930$ was only 1·75 ± 0·01 mm day^{-1} (strain 659-7, 30°C, pH 6·5). The behaviour of strain PR11 at 0·930 was also worth noting: at lower temperature, this strain out-performed strain 659-7 at this a_w. Only three strains (PR10, PR11, PR12) displayed residual growth at $a_w = 0·910$ (under some conditions) and no strain grew at $a_w = 0·880$.

At $a_w = 0·980$ for each strain and each tested pH, the growth rate appeared to decrease with decreasing temperature. At low a_w (0·930 and below), no clear conclusion emerges from the raw data regarding the effect of temperature. It is noteworthy that *Trichoderma* strains PR10, PR11, and PR12 proved more tolerant to low a_w at 20°C than at 30°C.

Table 2 Experimental and predicted values of radial growth rate of strains PR10, PR11, PR12, and 659-7 of *Trichoderma asperellum* obtained by applying factorial design (3^K) with temperature, water activity (a_w), and pH

	Environmental factors						Extension growth rate (mm day^{-1})							
	Experimental values			Coded values			Observed values				Predicted values			
	T°C	a_w	pH	T°C	a_w	pH	PR10	PR11	PR12	659-7	PR10	PR11	PR12	659-7
T_1	30	0·98	4·5	1	1	−1	11·49 ± 0·11	11·95 ± 0·03	14·80 ± 0·09	11·88 ± 0·02	10·75	11·99	14·17	12·1
T_2	30	0·98	6·5	1	1	0	9·44 ± 0·01	11·95 ± 0·00	14·70 ± 0·03	11·86 ± 0·06	9·98	11·78	13·52	11·46
T_3	30	0·98	8·5	1	1	1	9·23 ± 0·03	11·58 ± 0·03	11·58 ± 0·06	9·46 ± 0·04	8·90	11·43	12·12	9·86
T_4	30	0·93	4·5	1	0	−1	0·23 ± 0·02	0·91 ± 0·01	0·65 ± 0·01	1·25 ± 0·01	1·16	1·08	1·44	1·55
T_5	30	0·93	6·5	1	0	0	0·27 ± 0·02	0·29 ± 0·01	0·75 ± 0·01	1·75 ± 0·01	0·98	1·01	1·33	1·45
T_6	30	0·93	8·5	1	0	1	0·15 ± 0·03	0·29 ± 0·01	0·00 ± 0·00	0·00 ± 0·00	0·49	0·78	0·47	0·38
T_7	30	0·88	4·5	1	−1	−1	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00	0·00	0·00	0·00
T_8	30	0·88	6·5	1	−1	0	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00	0·00	0·01	0·12
T_9	30	0·88	8·5	1	−1	1	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00	0·00	0·00	0·00
T_{10}	25	0·98	4·5	0	1	−1	9·50 ± 0·05	11·48 ± 0·13	11·67 ± 0·07	11·47 ± 0·01	9·45	11·22	12·37	11·07
T_{11}	25	0·98	6·5	0	1	0	9·43 ± 0·01	11·55 ± 0·2	11·67 ± 0·03	11·45 ± 0·06	8·59	10·99	11·84	10·56
T_{12}	25	0·98	8·5	0	1	1	7·77 ± 0·04	11·13 ± 0·01	11·07 ± 0·06	9·36 ± 0·02	7·43	10·63	10·55	9·07
T_{13}	25	0·93	4·5	0	0	−1	0·39 ± 0·01	0·60 ± 0·07	0·12 ± 0·06	0·42 ± 0·18	0·89	0·92	0·53	1·09
T_{14}	25	0·93	6·5	0	0	0	0·33 ± 0·07	0·52 ± 0·03	0·23 ± 0·01	0·40 ± 0·08	0·62	0·83	0·54	1·11
T_{15}	25	0·93	8·5	0	0	1	0·19 ± 0·01	0·19 ± 0·07	0·14 ± 0·02	0·00 ± 0·00	0·05	0·60	0·00	0·15
T_{16}	25	0·88	4·5	0	−1	−1	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00	0·10	0·00	0·00
T_{17}	25	0·88	6·5	0	−1	0	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·29	0·14	0·10	0·34
T_{18}	25	0·88	8·5	0	−1	1	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·31	0·04	0·00	0·00
T_{19}	20	0·98	4·5	−1	1	−1	7·02 ± 0·06	9·69 ± 0·08	10·96 ± 0·03	9·51 ± 0·06	7·56	10·17	11·13	9·99
T_{20}	20	0·98	6·5	−1	1	0	6·78 ± 0·03	9·6 ± 0·01	10·91 ± 0·02	9·30 ± 0·03	6·62	9·93	10·7	9·60
T_{21}	20	0·98	8·5	−1	1	1	3·98 ± 0·02	8·74 ± 0·02	8·57 ± 0·01	7·64 ± 0·09	5·38	9·55	9·52	8·23
T_{22}	20	0·93	4·5	−1	0	−1	0·59 ± 0·03	1·26 ± 0·29	0·83 ± 0·03	1·15 ± 0·06	0·04	0·50	0·17	0·57
T_{23}	20	0·93	6·5	−1	0	0	0·51 ± 0·01	1·15 ± 0·01	0·80 ± 0·06	1·11 ± 0·17	0·00	0·39	0·29	0·71
T_{24}	20	0·93	8·5	−1	0	1	0·29 ± 0·04	1·04 ± 0·08	0·69 ± 0·06	0·81 ± 0·08	0·00	0·14	0·00	0·00
T_{25}	20	0·88	4·5	−1	−1	−1	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·16	0·29	0·08	0·00
T_{26}	20	0·88	6·5	−1	−1	0	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·39	0·32	0·73	0·51
T_{27}	20	0·88	8·5	−1	−1	1	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·32	0·20	0·63	0·21

All strains were able to grow at all pH levels tested (4·5, 6·5, and 8·5), provided the a_w was high enough. The growth rate was almost always higher at pH 4·5 and 6·5 than at 8·5. This effect of pH was clear at $a_w = 0·980$, diminishing with decreasing a_w. At low a_w values, it was practically absent.

Statistical analysis showed a significant effect ($P < 0·0001$) of a_w, incubation temperature, pH, and their interactions on the mycelial growth rates of *T. asperellum* strains PR10, PR11, PR12, and 659-7 (Table 3). Duncan's multiple-range test was performed to identify various homogeneous groups relating to each factor studied here and for each strain. For both strains PR10 and PR11, the test relating to the a_w factor revealed six homogeneous groups, one for each a_w value (data not shown). For strains PR12 and 659-7, this test distinguished respectively four and five homogeneous groups. For PR12, the first group consisted of $a_w = 0·995$ (control) and 0·980. The two following groups comprised respectively a_w values 0·960 and 0·930. The fourth group comprised the two lowest values of a_w. For 659-7, the four highest values of a_w formed four significantly different homogeneous groups while a_w values of 0·910 and 0·880 formed a common group (data not shown). Broadly, Duncan's multiple-range analysis confirmed that the optimal growth of *T. asperellum* strains ranged from 0·980 to 0·995 a_w. With regard to temperature, a similar test distinguished three statistically homogenous groups, one for each incubation temperature. Finally for pH, the test revealed three homogeneous groups for each strain corresponding to the three pH values.

Modelling the growth rate of *Trichoderma asperellum* strains

The modelling was performed to predict the behaviour of *T. asperlleum* strains under various environmental conditions. The obtained model will allow to understand the impact of each studied environmental factor on the survi-

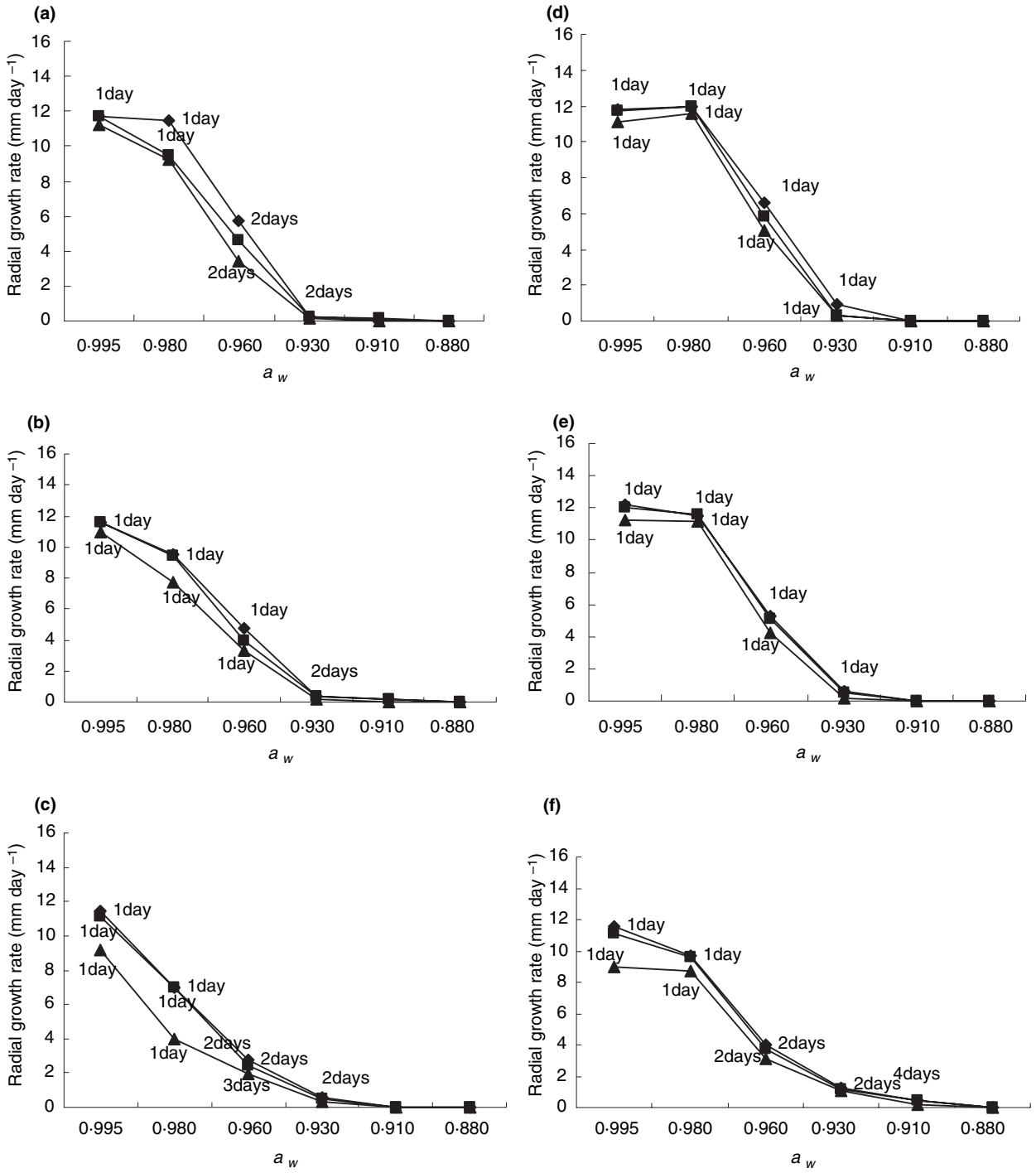

Figure 1 Effect of a_w on the radial growth rate of *Trichoderma asperellum* strain PR10 (a, b and c) and strain PR11 (d, e and f) at pH 4·5 (◆), 6·5 (■) and 8·5 (▲) and temperatures 30°C (a and d), 25°C (b and e) and 20°C (c and f). The number of days (d) for initiation of growth is shown.

val of *T. asperellum* strains and to identify the most influent one.

The average growth rates of the different strains obtained with the model for the various test conditions are listed in Table 2, along with the corresponding average experimental values.

The model fits the experimental data most closely under conditions where rapid growth is observed. In

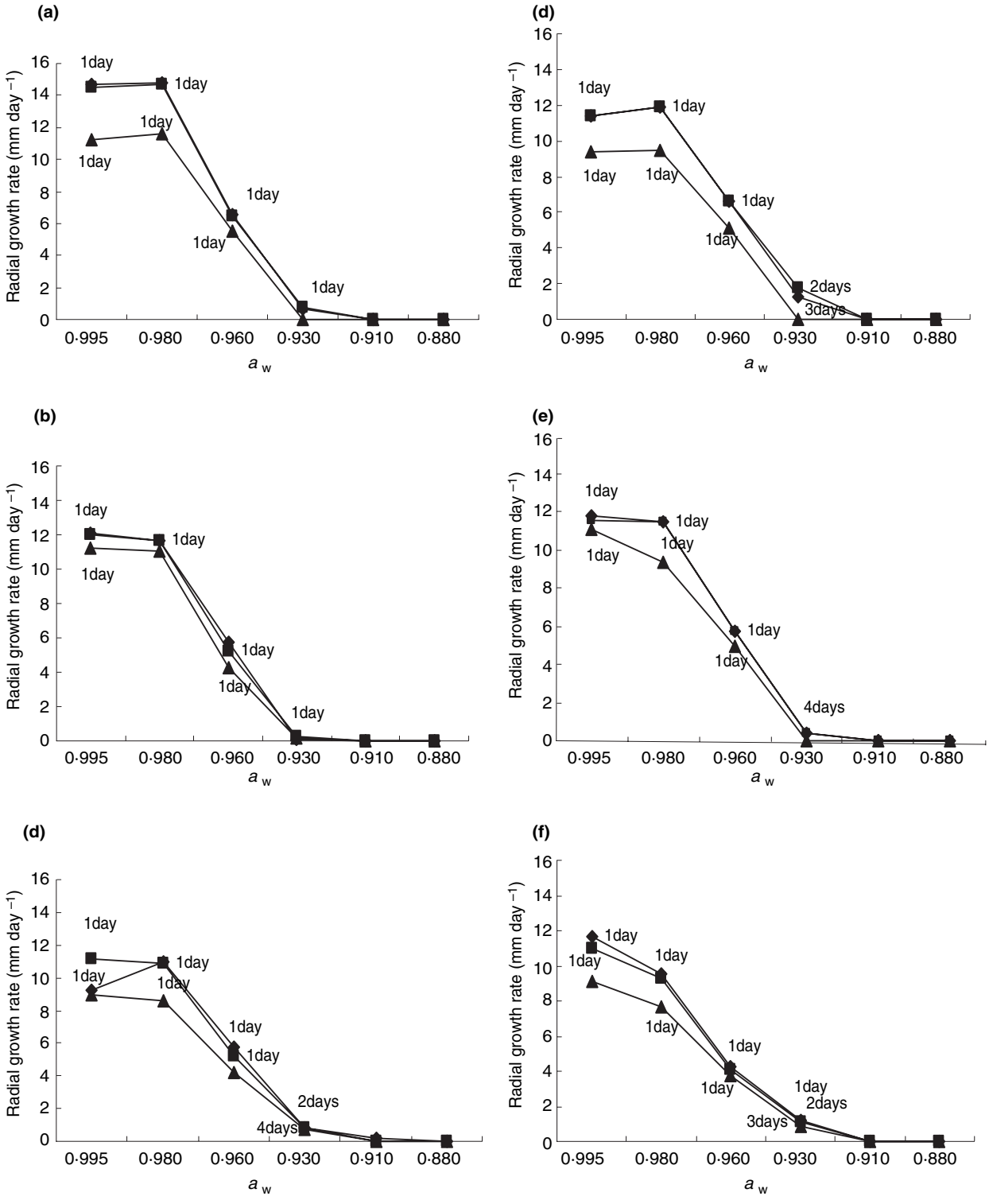

Figure 2 Effect of a_w on the radial growth rate of *Trichoderma asperellum* strain PR12 (a, b and c) and strain 659-7 (d, e and f) at pH 4·5 (♦), 6·5 (■) and 8·5 (▲) and temperatures 30°C (a and d), 25°C (b and e) and 20°C (c and f). The number of days (d) for initiation of growth is shown.

Table 3 Variance analysis of the effects of the water activity (a_w), temperature (T), pH, and two- and three-way interactions on the radial growth rate (mm day^{-1}) of four strains of *Trichoderma asperellum*

Strains	Source	*df*	MS	*F*	Pr > *F*
PR10	a_w	5	827·13	314597	<0·0001*
	T	2	28·02	10658·6	<0·0001*
	pH	2	14·60	5551·98	<0·0001*
	$a_w \times T$	10	9·24	3513·92	<0·0001*
	$a_w \times pH$	10	2·87	1090·68	<0·0001*
	$T \times pH$	4	0·64	244·38	<0·0001*
	$a_w \times T \times pH$	20	0·66	252·43	<0·0001*
PR11	a_w	5	997·76	307235	<0·0001*
	T	2	14·69	4522·56	<0·0001*
	pH	2	8·90	2740·03	<0·0001*
	$a_w \times T$	10	6·55	2017·11	<0·0001*
	$a_w \times pH$	10	0·98	302·14	<0·0001*
	$T \times pH$	4	0·22	67·83	<0·0001*
	$a_w \times T \times pH$	20	0·41	127·22	<0·0001*
PR12	a_w	5	44·91	96·89	<0·0001*
	T	2	1154·68	2491·20	<0·0001*
	pH	2	16·97	36·61	<0·0001*
	$a_w \times T$	10	11·20	24·17	<0·0001*
	$a_w \times pH$	10	2·03	4·39	·0022
	$T \times pH$	4	3·5	7·56	<0·0001*
	$a_w \times T \times pH$	20	0·94	2·03	·0086
659-7	a_w	5	926·96	282244	<0·0001*
	T	2	11·14	3391·11	<0·0001*
	pH	2	19·07	5807·54	<0·0001*
	$a_w \times T$	10	5·09	1550·65	<0·0001*
	$a_w \times pH$	10	2·91	887·03	<0·0001*
	$T \times pH$	4	0·84	256·22	<0·0001*
	$a_w \times T \times pH$	20	0·30	91·87	<0·0001*

*Highly significant at $P < 0·0001$.
df, degrees of freedom; MS, mean square.

particular, the highest predicted growth rate values occur at $a_w = 0·980$. This is in accordance with the experimental data: experiments 1, 2, 3, 10, 11, 12, 19, 20, and 21, showing the highest growth rates, are all characterized by $a_w = 0·980$, with various values of temperature and pH.

The model predicts adequately that the growth rate will decrease drastically, at all temperatures and pH levels, when the a_w is decreased to 0·960 and below. For the conditions corresponding to experiments 7, 8, 9, 16, 17, 18, 25, 26, and 27 ($a_w = 0·880$), where no growth was recorded experimentally, the model correctly predicts zero (or near-zero) values at 30°C, but its predictions tend to be too high at lower temperatures.

The model also predicts adequately, for each strain and each pH at $a_w = 0·980$, that the growth rate will decrease with decreasing temperature. It fails to show the growth rate increase observed at $a_w = 0·930$ when the temperature drops to 20°C, but it does predict such an effect at $a_w = 0·880$.

The results of the multiple regression analysis, which provided the estimated regression coefficients of the model for each *T. asperellum* strain, are shown in Table 4. The values of the coefficient of determination R^2 for each strain were 97·75, 99·18, 98·93, and 99·07 for PR10, PR11, PR12, and 659-7 respectively. For each strain, all regression coefficients were highly significant ($P < 0·0001$), except coefficients β_{11} (quadratic effect of temperature) and β_{13} (interaction effect between the temperature and the pH) for all strains and coefficient β_{33} (quadratic effect of the pH) for strains PR10 and PR11. Except for strain PR11, a significant interaction between pH and a_w was also demonstrated (β_{23}). The negative value of this coefficient suggests an antagonistic effect of the pH–a_w interaction on mycelial growth for strains PR10, PR12, and 659-7.

It appears that the higher the absolute value of the linear coefficient (β_2), the greater the influence of the corresponding factor (a_w) on the growth rate. The a_w has more influence than temperature or pH on the growth of *Trichoderma* strains. The linear effects of a_w and tempera-

Table 4 Model coefficients and their significant effects on the radial growth rate (mm day^{-1}) of strains PR10, PR11, PR12, and 659-7 of *Trichoderma asperellum*

R^2	Coefficients	PR10 97·75	PR11 99·18	PR12 98·93	659-7 99·07
Response means	β_0	0·62***	0·83***	0·54***	1·11***
T	β_1	0·65***	0·05***	0·52***	0·37***
a_w	β_2	4·15***	5·43***	5·87***	5·11***
pH	β_3	–0·42***	–0·16***	–0·37 ***	–0·47***
T^2	β_{11}	$-0·29^{ns}$	$-0·14^{ns}$	$0·27^{ns}$	$-0·03^{ns}$
a_w^2	β_{22}	3·82***	4·73***	5·43***	4·34***
pH^2	β_{33}	$-0·15^{ns}$	$-0·07^{ns}$	–0·37***	–0·49***
$T \times a_w$	β_{12}	1·03***	0·62***	0·89***	0·56***
$T \times pH$	β_{13}	$0·08^{ns}$	$0·01^{ns}$	$-0·11^{ns}$	$-0·12^{ns}$
$a_w \times pH$	β_{23}	–0·58***	$-0·14^{ns}$	–0·54***	–0·53***

***Significant ($P < 0·0001$).
ns, non significant ; a_w, water activity.

ture on mycelial growth appear to be positive. In contrast, pH has a negative linear effect.

Discussion

The objective of this work was to determine the influence of the principal environmental factors on the growth of strains PR10, PR11, PR12, and 659-7 of *T. asperellum*, used as biological control agents against *P. megakarya*, the causal agent of cocoa black pod disease. The present *in vitro* experiments were intended to provide a baseline evaluation of the behaviour of *T. asperellum* under optimal nutritional conditions, although these are certainly not found in the field. We focused on the growth rate as a possible indicator of the capacity of *T. asperellum* to colonize the cocoa ecosystem.

Few data are available in the literature on the combined effects of environmental factors on the growth of *T. asperellum.* Our statistical analyses of the responses observed on PDA medium show that a_w, temperature, and pH are key determinants of the development of *T. asperellum* strains. Among these, a_w has the greatest influence on the growth of strains. This tallies with the finding that the amount of available water in the substrate and the surrounding environment are very important to ensure fungal growth (Verscheure *et al.* 2002). For all of the studied strains of *Trichoderma*, growth was fastest at a_w between 0·995 and 0·980. These *T. asperellum* strains appear to be very sensitive to a decrease in the a_w of the medium. Our results are in agreement with those reported by Kredics *et al.* (2000, 2004), who observed limited growth of *Trichoderma* spp. at $a_w = 0{\cdot}92$. One of the major limitations of the use of *Trichoderma* species as biofungicides is their low level of osmotolerance (Kredics *et al.* 2003). To ensure the growth of propagules, it is necessary to add a substrate that maintains a constant high a_w (Wakelin *et al.* 1999).

Our results show that the studied *Trichoderma* strains can grow at all temperatures and all pH levels tested (20–30°C, pH 4·5–8·5). Growth of all strains of *T. asperellum* was fastest at 30°C, as reported by Samuels *et al.* (1999), and at pH = 4·5–6·5. Similar results have been reported for other *Trichoderma* species. Kredics *et al.* (2004) reported that *Trichoderma harzianum*, *Trichoderma aureoviride*, and *Trichoderma viride* can grow over a broad pH range (from 2·0 to 6·0), with optimal growth at pH = 4·0. The fact that our strains grew more slowly at 20–25°C and at low a_w may explain the inconsistent performances of mycoparasitic *T. asperellum* strains when used on cocoa plantations in Cameroon, where the growing period is marked by fluctuations in relative humidity and where the average minimum and maximum daily temperatures recorded under cocoa trees are about 20 and 26°C, respectively (Ndoumbè-Nkeng *et al.* 2004). Under these conditions, field test results showed strain PR11 to be most effective at all locations (Tondje *et al.* 2003). In our study likewise, PR11 displayed faster growth than the other strains at 20°C and low water activity. This relative resistance to stress may favour better colonization and faster establishment of the antagonistic population, and thus higher effectiveness. Nevertheless, as *Trichoderma* strains are considered as mycoparasitic, a differential production of hydrolytic enzymes between four strains depending on a_w value, may not be excluded. This is also valid for other modes of action, like antibiosis. Consequently, the better biological performance of strain PR11 may also be explained by the relative contribution of other modes of action.

The necessity to consider physical factors for efficient growth of *T. asperellum* strains makes it useful to develop a mathematical model for predicting strain behaviour under various environmental conditions. Our multifactorial analysis shows that the quadratic polynomial model provides a good approach to predict the combined effects of a_w, temperature, and pH on the radial growth rate of *T. asperellum* strains. The predictions of this model correlate largely with the experimental results, although the match seems better at higher growth rates than at very low ones. A similar approach has been used to predict the growth of food spoilage pathogens such as *Penicillium chrysogenum* and *Aeromonas hydrophila* (Sautour *et al.* 2001, 2003), *Rhizopus oligosporus* NRRL2710 (Sparringa *et al.* 2002), and of stored fruit pathogens like *Penicillium expansum* (Lahlali *et al.* 2005). The approach has also been used to predict the growth and sporulation of *Bacillus subtilis* 355 and the production of antifungal metabolites by this bacterium (Moita *et al.* 2005).

The growth rates predicted by the model clearly reflect the strong dependence of *T. asperellum* growth on the a_w of the medium at any temperature and pH. Yet, a_w is not the only environmental factor that can affect the growth of living organisms, and each separate factor is liable to influence the effect exerted by other factors (Sparringa *et al.* 2002). Accordingly, interactions between the environmental factors tested appear to influence the growth of the tested *T. asperellum* strains in this model.

Significant interactions were revealed between a_w and temperature on the one hand and between a_w and pH on the other. At $a_w = 0{\cdot}880$, the model predicts some, albeit slight growth at 20–25°C, but zero (or near-zero) growth at 30°C. Experimentally, no growth was observed at $a_w = 0{\cdot}880$, but at $a_w = 0{\cdot}910$ and 0·930, some strains did show better growth at 20°C than at higher temperature. These results suggest that *T. asperellum* strains may support some stresses better when the temperature is lower.

In our study, glycerol was the solute used to reduce a_w. This solute can support growth because of its potential as a carbon source for micro-organisms (Parra *et al.* 2004). In addition, glycerol exerts no inhibitory effect (Baxter *et al.* 1998) and can be used to reach lower a_w values than with NaCl (Kredics *et al.* 2004; Lahlali *et al.* 2005).

Modelling the growth of *T. asperellum* strains on a solid substrate is a first step towards predicting the behaviour of such strains under field conditions and towards finding a formulation that takes their ecophysiological features into account so as to permit their successful use in the field. The results of this first approach suggest that humectants might be used to increase the amount of available water for biological control agents. Yet, one should bear in mind that the present model, developed in a specific experimental framework, might not remain valid outside that framework (Delignette-Muller 1997). Any extrapolation to natural situations and beyond the tested ranges of the considered factors is risky, as other factors such as ultraviolet radiation, nutrient availability, and interactions with other micro-organisms on the fruit surface may modify the behaviour of *Trichoderma* strains. For this reason, other studies are necessary to develop experimental protocols for assessing the effects of environmental factors on the growth of *T. asperellum* strains on the fruit surface and to validate models under *in vivo* conditions. The development of a model describing the population dynamics of *P. megakarya* in the field would contribute greatly to develop an effective formulation for a biofungicide based on *T. asperellum*.

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