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# *In vitro* study of the influence of temperature, pH, and $a_w$ on the growth rate of *Trichoderma asperellum*

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**Abstract:** The effects of water activity ($a_w$), temperature and pH were evaluated on the radial growth of *Trichoderma asperellum* (strains PR10, PR11, PR12, 659-7), an antagonist of *Phytophthora megakarya*, the causal agent of cocoa black pod disease. The radial growth of four strains of *T. asperellum* was monitored for 30 days on PDA modified medium at six levels of $a_w$ (0.995-0.880), three values of pH (4.5, 6.5 nd 8.5) and three incubation temperatures (20, 25 and 30°C). Whatever the strain, mycelial growth rate was optimal at water activities between 0.980 and 0.995, independently of incubation temperature and pH. All strains appeared to be very sensitive to $a_w$ reduction. In addition, all four strains were able to grow at all temperatures and pH values (4.5-6.5), highest growth being observed at 30°C and pH= 4.5-6.5.

**Keyword:** radial growth rate, water activity

## Introduction

The cocoa tree (*Theobroma cocoa* 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 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). *P. 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). Classically, cocoa growers use farming strategies, genetic strategies, and most often chemical strategies to minimise the impact of black pod disease (Akrofi et al., 2003).

Biological control by means of antagonistic microorganisms is an emerging strategy in many countries affected by this disease (Krauss & Soberanis, 2001). In Cameroon, research focusing on biological control of *Phytophthora megakarya* began in 1999. It has led to the isolation and identification of mycoparasitic strains of *Trichoderma asperellum* (Tondje et al., 2005).

Currently these *T. asperellum* strains (PR10, PR11, PR12, and 659-7) are applied under natural conditions in cocoa fields but the level of protection afforded by such biocontrol agent preparations has been inconsistent. The variable performance of *T. asperellum* as a biocontrol agent could be due to the influence of environmental factors that vary in time and from farm

to farm. No literature is available concerning the effect of these environmental parameters on the development of *T. asperellum*. In this context, the main objective of this work was to determine *in vitro* effects of temperature, pH and $a_w$ on the radial growth rate of *T. asperellum* strains (PR10, PR11, PR12, 659-7).

## Materials and methods

*Microorganisms*

Four strains of *T. asperellum* (PR10, PR11, PR12 and 659-7) were used in this study. These strains were isolated from tubers of *Xanthosoma* spp. and *Musa spp.* in a mixed crop field around Yaoundé in Cameroon and then stored on PDA medium at 4°C.

*Medium*

The basic medium used was Potato Dextrose Agar (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 mM $NaH_2PO_4$, the final pH of the medium being adjusted to pH 4.5 with 80% $H_3PO_4$ or to pH 6.5 or 8.5 with 1 M NaOH before autoclaving. The $a_w$ of all 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 ml 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 cheesecloth. Suspensions were adjusted to $10^6$ conidia/ml using a Bürker cell, then 10-µl aliquots of suspension were inoculated at the centre of Petri dish containing test medium. After inoculation, the Petri plates were sealed in polyethylene, and then incubated for 30 days at 20, 25 or 30°C.

*Data recording*

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

*Statistical analysis*

Growth rates were subjected to the general linear model (GLM) procedure of the Statistical Analysis System (SAS). Statistical significance was performed at the $P \leq 0.05$ level. Where analysis revealed significant differences, Duncan's multiple range test for separation of means was performed.

## Results

Statistical variance analysis of the data provided a highly significant effect ($P<0.0001$) of $a_w$, incubation temperature, pH and their two and three interactions on mycelial growth rates of *T. asperellum* strains PR10, PR11, PR12 and 659-7 (results not shown). Duncan's multiple range test was performed to distinguish the various homogeneous groups relating to each factor studied here and for each strain (Figs 1-3). Regardless the strain, the growth rate was significantly correlated to the water activity of the medium. The highest value was observed at $a_w$ of 0.980, independently of incubation temperature and pH level. At $a_w$ of 0.910, strains PR10, PR11 and PR12 displayed a residual growth under some conditions. No growth was observed at $a_w$ of 0.880 whatever the strain.

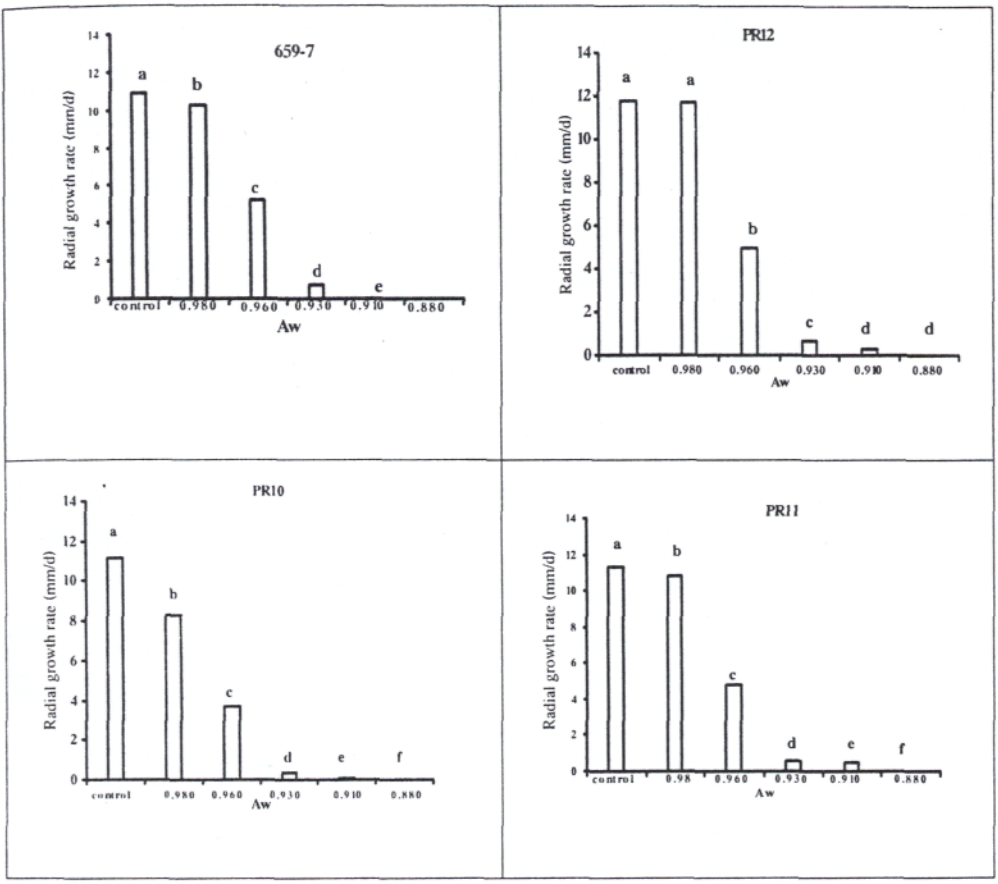


Figure1. Effect of water activity ($a_w$) on the radial growth rate of *T. asperellum strains*

At 30°C, the growth rates were highest for all strains. At 20°C, the growth rate was lowest as compared to 25-30°C. However no growth was observed at $a_w$ of 0.910 $a_w$ for strains PR10 and 659-7 whatever the level of pH tested. Also, a very low residual growth was observed for strain PR11. All strains were able to grow at all pH levels (4.5, 6.5 and 8.5). The growth rate was almost always higher at pH 4.5 for PR10, PR11 and 659-7 and at pH 6.5 for PR12 than pH 8.5.

## Discussion

The statistical analysis of radial growth rate of *T. asperellum* showed that the environmental factors ($a_w$, temperature and pH) are a key elements could limited the development of the *T. asperellum* strains. The $a_w$ factor has the greatest influence on the growth rate of *T. asperellum* strains. The optimal growth was observed at $a_w$ ranging from 0.980 to 0.995. *T. asperellum strains* were shown to be highly sensitive to the dropped of $a_w$ of the medium. These results are in agreement with those reported by Kredics et al. (2004) who observed limited growth of *Trichoderma spp* at 0.92 $a_w$. Subsequently, applying *Trichoderma* strains as

biological control agent must be supported by a substrate that maintains a constant high $a_w$ to allow the growth of the propagules (Wakelin et al., 1999).

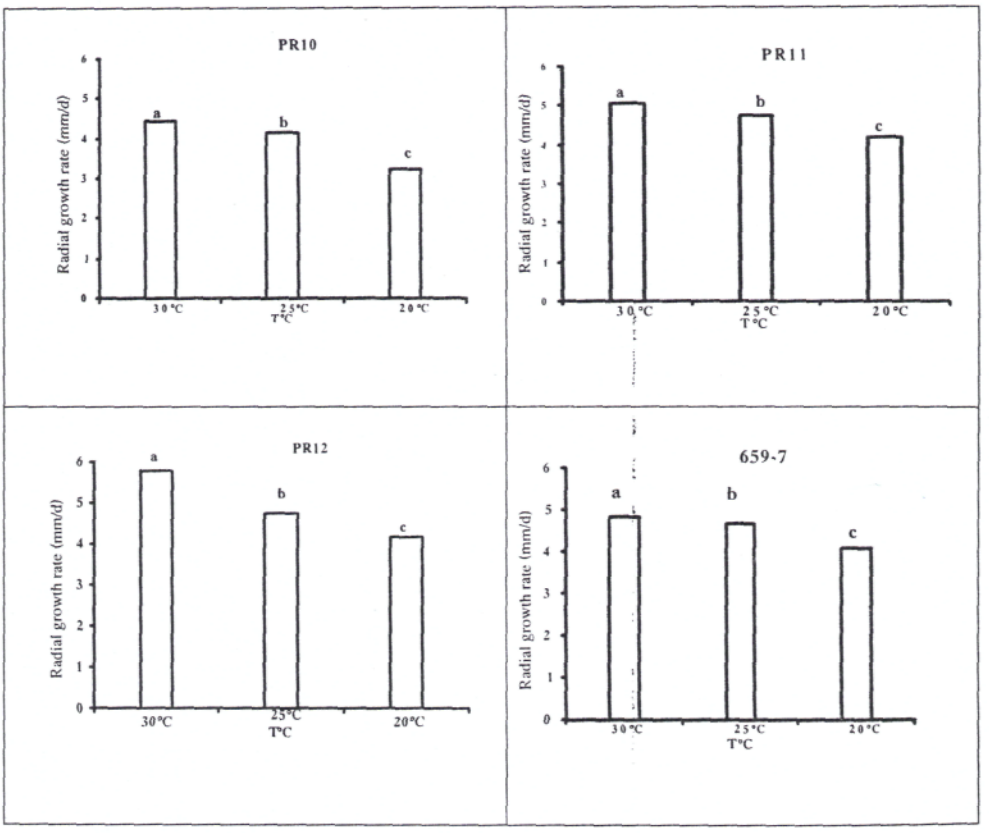


Figure 2. Effect of temperature on radial growth rate of *T. asperellum strains*

Our results indicated that the growth of the studied strains of *Trichoderma* was possible at all tested levels of temperatures and pH ranging from 4.5 to 8.5. The highest growth of *T. asperellum* strains was observed at incubation temperature of 30°C as previously reported by Samuels et al. (1999), and at pH values between 4.5 and 6.5. Similar results were found on other species of *Trichoderma*. Kredics et al. (2004) reported that *T. harzianum, T. aureoviride* and *T. viride* were able to grow within a broad range of pH ranging from 2.0 to 6.0 with an optimal growth at pH = 4.0. The lowest growth rate of the studied *T. asperellum* strains was recorded between 20 and 25°C at lower values of $a_w$. This result seems explained the inconsistent performances of the mycoparasitic strains of *T. asperellum* when they are applied in cocoa plantations in Cameroon. In fact, in cocoa plantations, one observes throughout the growing period, fluctuations of relative humidity and 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 location (Tondje et al., 2005). In our study likewise, PR11 displayed faster

growth than other strains at 20°C and low water activity (0.930). This resistance to stress may favour better colonization and faster establishment of the antagonistic population, and thus better effectiveness.

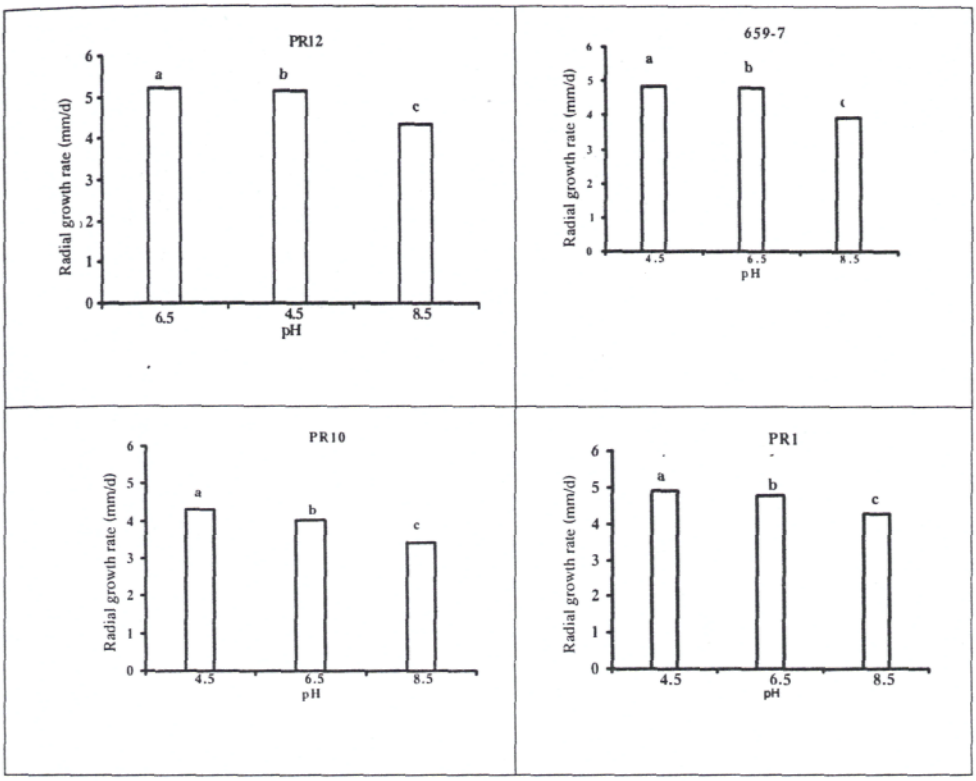


Figure 3. Effect of pH on the radial growth rate of *T. asperellum*

The results from the assessment of the effects of environmental factors such as temperature, $a_w$ and pH on the growth of *T. asperellum* strains on solid substrate is a first investigation making it possible in the future to predict the behaviour and to handle the ecophysiological aptitudes in order to develop an effective formulation for a biofungicide based on *T. asperellum*.

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## References

Akrofi, A.Y., Appiah, A.A. & Opoku, I.Y. 2003: Management of Phytophthora pod rot disease on cocoa farms in Ghana. Crop Prot. 22: 469–477.

Appiah, A.A., Flood, J., Archer, S.A. & Bridge, P.D. 2004: Molecular analysis of isolates of *Phytophthora spp* responsible for black pod disease of cocoa using RFLPs, mitochondrial DNA haplotyping and AFLP. Proc of the INCOPED 4th Intern. Sem. Accra, 19th – 21st oct 2003.

ICCO (International Cocoa Organisation). 2003: Rapport annuel 2002/2003.22 Berners Street, Londres W1P 3DB: 31 pp.

Kredics, L., Manczinger, L., Antal, Z., Pénzes, Z., Szekeres, A., Kevei, F. & Nagy, E. 2004: *In vitro* water activity and pH dependence of mycelial growth and extracellular enzyme activities of *Trichoderma* strains with biocontrol potential. J. Appl. Microbiol. 96: 491-498.

Lahlali, R., Friel, D. & Jijakli, M.H. 2006: *In vitro* effects of water activity, temperature and solutes on the growth rate of *P. italicum* Whmer and *P. digitatum* Sacc. J. Appl. Microbiol. 101: 628-636.

Ndoumbè-Nkeng, M., Cilas, C., Nyemb, E., Nyasse, S., Bieysse, D., Flori, A. & Sache, I. 2004: Impact of removing diseased pods on cocoa black pod caused by *Phytophthora megakarya* and on cocoa production in Cameroon. Crop Prot. 23: 415-424.

Samuels, G.J., Lieckfeldt, E. & Nirenberg, H.I. 1999: *Trichoderma asperellum*, a new species with warted conidia, and redescription *T. viride*. Sydowia 51: 71-88.

Tondje, P.R., Begoude, B.A.D., Samuels, G.J., Hebbar, P.K. & Foko, J. 2005: Biological Control of the cocoa black pod disease causative agent *Phytophthora megakarya* with *Trichoderma asperellum*. 14th International Cocoa Research Conference, VOL. II, pp. 677-682 (Accra- Ghana 13-18 October 2003) *Cocoa producers' alliance Lagos Nigeria* (eds).

Wakelin, S.A., Sivasithamparam, K., Cole, A.L.J. & Skipp, R.A. 1999: Saprophytic growth in soil of a strain of *Trichoderma koningii*. NZ J. Agric. Res. 42: 337-345.