Effects of Temperature and Relative Humidity on the In Vitro and In Vivo Radial Growth of *Penicillium italicum* and on the Biocontrol Activity of *Pichia guilliermondii*, Strain Z1

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Abstract

The objective of this study was to assess the effect of temperature (5-25°C) on the 'in vitro' and 'in vivo' growth rates of *Penicillium italicum* and to determine the combined effect of temperature and relative humidity (45 to 100%) on lesion size of this pathogenic fungus on Valencia late oranges, either alone or in combination with the antagonistic yeast strain Z1 of *Pichia guilliermondii* Wickerham. Statistical analysis showed a significant effect of temperature on the 'in vitro' and 'in vivo' radial growth of *P. italicum* with the maximum growth observed at temperature of 25°C. In both cases, no growth was observed at a temperature of 35°C. These factors had a significant effect on *P. italicum* lesion size when it was applied alone on Valencia late oranges and insignificant when yeast strain Z1 was applied 24 h before *P. italicum* inoculation. Our results confirm previous 'in vitro' findings that a_w has a greater influence than temperature on *P. italicum* growth and highlight that the strain Z1 showed high antagonistic potential against this pathogen over a range of temperature-relative humidity regimes favouring *P. italicum* development.

INTRODUCTION

Blue mold, caused by *Penicillium italicum* Wehmer, is considered as one of the main destructive postharvest diseases of citrus worldwide (Zhang et al., 2005; Caccioni et al., 1998; Homes and Ekert, 1999; Palou et al., 2002). This wound pathogen is more common in fruit held in cold storage during summer; it has a relatively short disease cycle (3-5 days at 25°C). A single fruit, can produce 1 to 2×10^9 conidia that are efficiently dispersed via air currents (Homes and Eckert, 1995). To date, this disease is primarily controlled by application of synthetic fungicides such as imazalil, thiabendazole. Currently there are no alternatives to these postharvest chemicals in the citrus industry. Biological control of postharvest decays of fruit and vegetables has been an active research area and may provide a good alternative to chemicals (Droby and Chalutz, 1999; Wilson et al., 1991). Biocontrol may also provide a good tool to solve the problem of chemical residues in fruit and pathogen resistance development. Compared with root or foliar diseases, postharvest disease is more suitable for biocontrol since the environmental factors are more stable and can be controlled. In this context, a yeast strain (Z1) of *Pichia guilliermondii* was previously selected from Moroccan Valencia late oranges for its antagonistic activity against *P. italicum* and its efficacy was proved in semi-commercial trials (El Guilli et al., 2009).

The importance of incidence attacks of *P. italicum* in orange fruits during storage required an understanding of its ecological behaviour and particularly about its interactions with the microbial flora present on the fruit surface (Lacey, 1989), in order to develop more rational strategies. Environmental factors such as, water activity, relative humidity and temperature are the most important factors influencing spore germination and growth of propagules on the fruit surface (Magan and Lacey, 1988; Plaza et al.,

2003). Recently, Lahlali et al. (2006) have developed four models predicting the in vitro radial growth rate of this pathogenic fungus related to water activity of the medium and incubation temperature. Optimal growth was observed at a_w ranging from 0.96 to 0.98 and at a temperature of 25°C. However, little information is available on how relative humidity and temperature affect the radial growth of *P. italicum* in vitro and on the citrus fruit surface. In this context, the main objectives of this work were to evaluate the effect of temperature and relative humidity on the growth rates of *P. italicum* in vitro and in vivo conditions and to assess the combined effect of both factors on its biological control efficacy.

MATERIALS AND METHODS

Strains

The strain of *Penicillium italicum* used in this study was originally isolated from decayed Valencia late orange fruits from the Gharb region of Morocco. For long term storage, the pathogen was maintained in 25% glycerol at -80°C. The fungus was recovered from glycerol and grown on potato dextrose agar (PDA) when it was needed prior to each experiment.

Yeast strain Z1 was isolated from healthy Moroccan Valencia late oranges by the laboratory of Phytopathology of INRA-El Menzeh and identified as *Pichia guilliermondii*. It was selected for its high and reliable protective activity against *P. italicum* and *P. digitatum*. Before use, strain Z1 was cultured at 25°C for 3 successive generations on PDA medium with an interval of 24 h. The final concentration was determined by spectrometer.

Fruits Preparation

Valencia late orange fruits were disinfected by soaking during two min in sodium hypochlorite (10%) then rinsed twice in sterile distilled water. After drying for one hour, oranges were wounded in two equidistant points at the equatorial site. Each wound was 5 mm in diameter and 4 mm in deep.

The Effect of Temperature on In Vitro and In Vivo Growth of *P. italicum*

A 10-day-old colony culture of *P. italicum* grown on PDA was used to obtain spore suspensions in sterile distilled water containing 0.05% Tween 80. Spore suspensions were adjusted to 1×10^6 spores/ml using a Bürker cell. A 10 µl aliquot of this suspension was inoculated at the centre of Petri dishes. After inoculation, the Petri plates were sealed and incubated at different temperatures (5, 10, 15, 20, 25 and 35°C).

The radial mycelial growth of each growing colony was measured daily in two perpendicular direction for temperatures ranging from 15 to 35°C and every two days for 5 and 10°C., without opening the Petri dishes. Observations were recorded until the plates were completely colonized (Lahlali et al., 2006; Parra and Magan, 2004). The radial mycelial growth was plotted and radial growth rates (mm d^{-1}) were estimated for each temperature from linear regression slopes of temporal growth curves.

Regarding the effect of temperature on *P. italicum* growth in vivo, Valencia late oranges were disinfected and wounded as described above. Wounded fruits were inoculated with *P. italicum* with the same suspension (10 µl per wound) at 10^6 spores/ml. Treated fruits were incubated at the various test temperatures. Lesion diameter of *P. italicum* was measured daily in two perpendicular direction for temperatures ranged from 15 to 35°C and every two days at temperatures of 5 and 10°C. Four replicates per treatment were done. In order to compare the in vitro and in vivo obtained results, an estimate of the radial growth rate of *P. italicum* on Valencia late oranges was calculated using a regression method. In both experiments, a quadratic polynomial (Y (mm d^{-1}) = a T^2 + b T + c where a, b and c are the regression coefficients) equation was used to fit the radial growth of blue mold.

Combined Effect of Temperature and Relative Humidity on Lesion Size of *P. italicum* and Its Biological Control by *P. guilliermondii* (Z1)

Fruits were prepared as described previously. Fifty µl of strain Z1 at 1×10^8 cfu/ml were applied on wounded fruits. Fifty µl of sterile distilled water were applied on the control before *P. italicum* inoculation. Twenty four hours after yeast application, wounded fruits were inoculated with 50 µl of *P. italicum* at concentration of 1×10^5 spores/ml. The inoculated fruits were kept at different temperatures 5, 10, 15, 20, 25 and 35°C at different relative humidity regimes 45, 75, 85, 98, and 100%. To control humidity, desiccators were used. RH inside desiccators was monitored using saturated salt solutions: K_2CO_3 (45%), NaCl (75%), KCl (86.5%) and K_2SO_4 (98%). The experiment lasted 30 days for the temperatures ranging from 5 to 15°C, and only 8 days for temperatures ranging from 20 to 35°C.

Statistical Analysis

The single effect of temperature on the in vitro and in vivo radial growth rate (mm d^{-1}) of *P. italicum* was performed by ANOVA one way of SAS software (SAS Institute, version 8.2, Cary, NC, USA). However, to assess the combined effect of temperature and relative humidity on lesion size of *P. italicum*, a full randomized factorial design was used to analyze the lesion size of this pathogenic fungus on Valencia late oranges at different temperature and relative humidity regimes. The effect of temperature, relative humidity, and their interaction was performed using a general linear model (GLM) procedure of SAS software. In both experiments, when results were statistically significant, a Duncan's multiple range test was employed for mean separation.

RESULTS AND DISCUSSION

Temperature Effect on the In Vitro and In Vivo *P. italicum* Growth

In both in vitro and in vivo experiments, temperature had a significant effect on the radial growth rate of *P. italicum* (Fig. 1). The highest in vitro growth was observed at a temperature of 25°C (Fig. 1a). This result was in agreement with that reported by Lahlali et al. (2006) and Plaza et al. (2003) who found that the optimum growth of *P. italicum* was at 25°C. Growth was greatest at temperature ranging from 20 to 25°C on Valencia late oranges (Fig. 1b). At any specific temperature, the growth was greatest in vivo compared to growth in vitro.

In the present study, no growth of *P. italicum* was observed at temperature of 35°C in vitro and in vivo conditions which suggests that the maximum temperature for growth ranged between 25 and 35°C. Plaza et al. (2003) reported that 30°C was the maximum temperature for growth of *P. italicum*; they detected no growth of this food borne pathogen at temperature of 37°C.

Generally, whatever the studied conditions, growth rates increased with an increase in temperature and the optimum temperature for growth was observed at 25°C, as previously reported by Lahlali et al. (2006), Plaza et al. (2003) and Lacey (1989). The effect of temperature on the in vitro and in vivo radial growth rates of *P. italicum* can be fitted by the following quadratic equations respectively: $-0.014\ T^2 + 0.56\ T-1.36$ and $-0.017\ T^2 + 0.637\ T-0.33$).

Combined Effect of Temperature and Relative Humidity on Lesion Size of *P. italicum* and Its Biocontrol

Statistical analysis of variance (ANOVA) showed a highly significant effect of temperature, relative humidity and their interaction on the lesion size of *P. italicum* on Valencia late oranges (Table 1). Lesion size decreased with decreasing temperature and relative humidity values, except at temperatures of 10 and 15°C where a similar lesion size was observed in both relative humidity treatments 85 and 98%. Lesion size was greatest at 25°C and a relative humidity of 100% (Fig. 2a). This result is in concordance with in vitro findings (Lahlali et al., 2006). No growth was detected at temperature of

35°C regardless of the relative humidity value.

In the present work, it seems that relative humidity had a greater effect than temperature on the lesion size of *P. italicum* (Table 1). Lahlali et al. (2006) modeled the in vitro effect of temperature and a_w on radial growth of this pathogen and found that a_w has a more pronounced effect than temperature.

Figure 2b shows that antagonistic yeast strain Z1 of *P. guilliermondii* significantly suppressed lesion formation of *P. italicum* under most temperature and relative humidity combinations. Moreover, the effect of the these factors and their interaction were insignificant (Table 2). This indicates that the control efficacy achieved by with Z1 was independent of the environmental conditions as previously reported by Guetsky et al. (2001) against *B. cinerea*. This result could be explained by the environment of the wound which may protect the antagonistic yeast against from the adverse effects of the environmental factors studied here or alternatively by the time lapse between biocontrol agent application and pathogen inoculation. However, it has been demonstrated by several researchers that the effect of these factors could be significant if the yeast is applied directly on the fruit surface (Bonaterra et al., 2006; Lahlali, 2006).

CONCLUSIONS

This is the first study which has tried to determine the combined effect of temperature and relative humidity on *P. italicum* growth rate and on the ability of the Z1 yeast to exhibit biocontrol activity and to compare the obtained results with our previous in vitro findings regarding the effect of ecological factors like temperature and a_w on the in vitro growth of this pathogenic fungus. The in vivo and in vitro growth decreased with decreasing temperature of incubation as previously reported by several investigators (Lahlali et al., 2006; Plaza et al., 2003; Parra and Magan, 2004). In our work we also found that the *P. italicum* grows faster on citrus fruit than on PDA agar plates at all temperature and relative humidity values.

In summary, our results highlight that the growth of *P. italicum* is significantly dependent on temperature and relative humidity and the effect of relative humidity seems to be greater than that of temperature. In contrast, the efficiency obtained with the antagonistic yeast strain Z1 was independent of temperature-relative humidity regimes. This result indicates that yeast strain Z1 has a strong potential as a postharvest biocontrol agent for Valencia late oranges storage, as its effectiveness for the protection of wounded fruits was high over a range of temperature-relative humidity regimes allowing the development of blue mould.

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Tables

Table 1. Analysis of variance of the effect of temperature (T), relative humidity (RH) and their interaction on the lesion size of *P. italicum*.

Source of variation	*DF*	SS	MS	F
T	4	539.57	134.89	17.14*
RH	4	2082.19	520.54	66.16*
T x RH	16	521.26	32.57	4.14*
Error	75	590.08	7.86	

DF: degree of freedom; SS: sum of squares, MS: mean square.
* Highly significant P<0.0001.

Table 2. Analysis of variance of the effect of temperature (T), relative humidity (RH) and their interaction on lesion size of *P. italicum* in presence of yeast strain Z1.

Source of variation	*DF*	SS	MS	F
T	4	0.54	0.13	0.59^{NS}
RH	4	2.26	0.56	2.45^{NS}
T x RH	16	6.27	0.39	1.70^{NS}
Error	75	590.08	7.86	

NS: No significant.

Figures

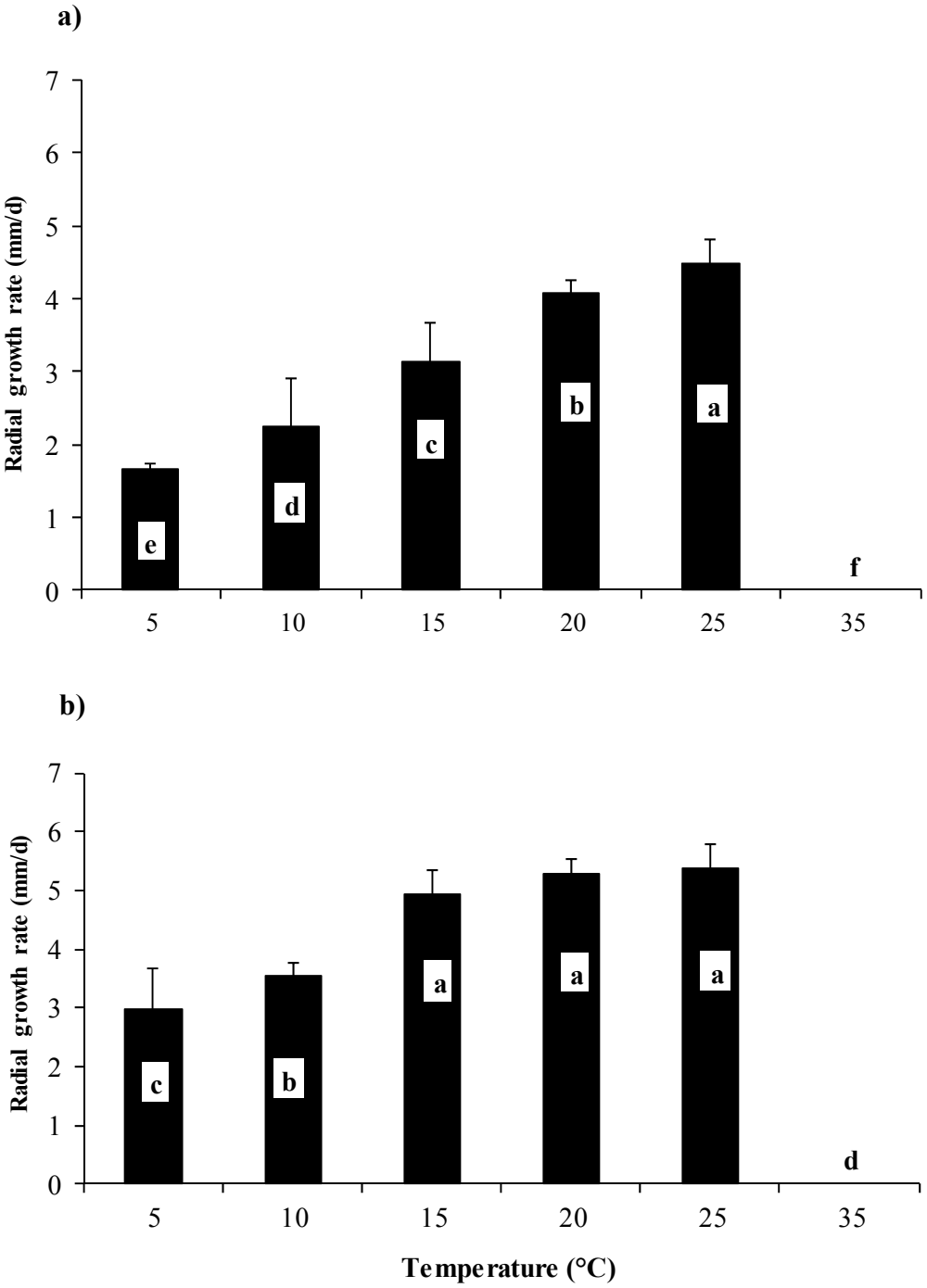

Fig. 1. Effect of temperature on radial growth rate of *P. italicum* grown on PDA medium (a) and on Valencia late oranges. Bars represent the standard error of the mean values. Treatments having the same letters are not significantly different ($P<0.05$).

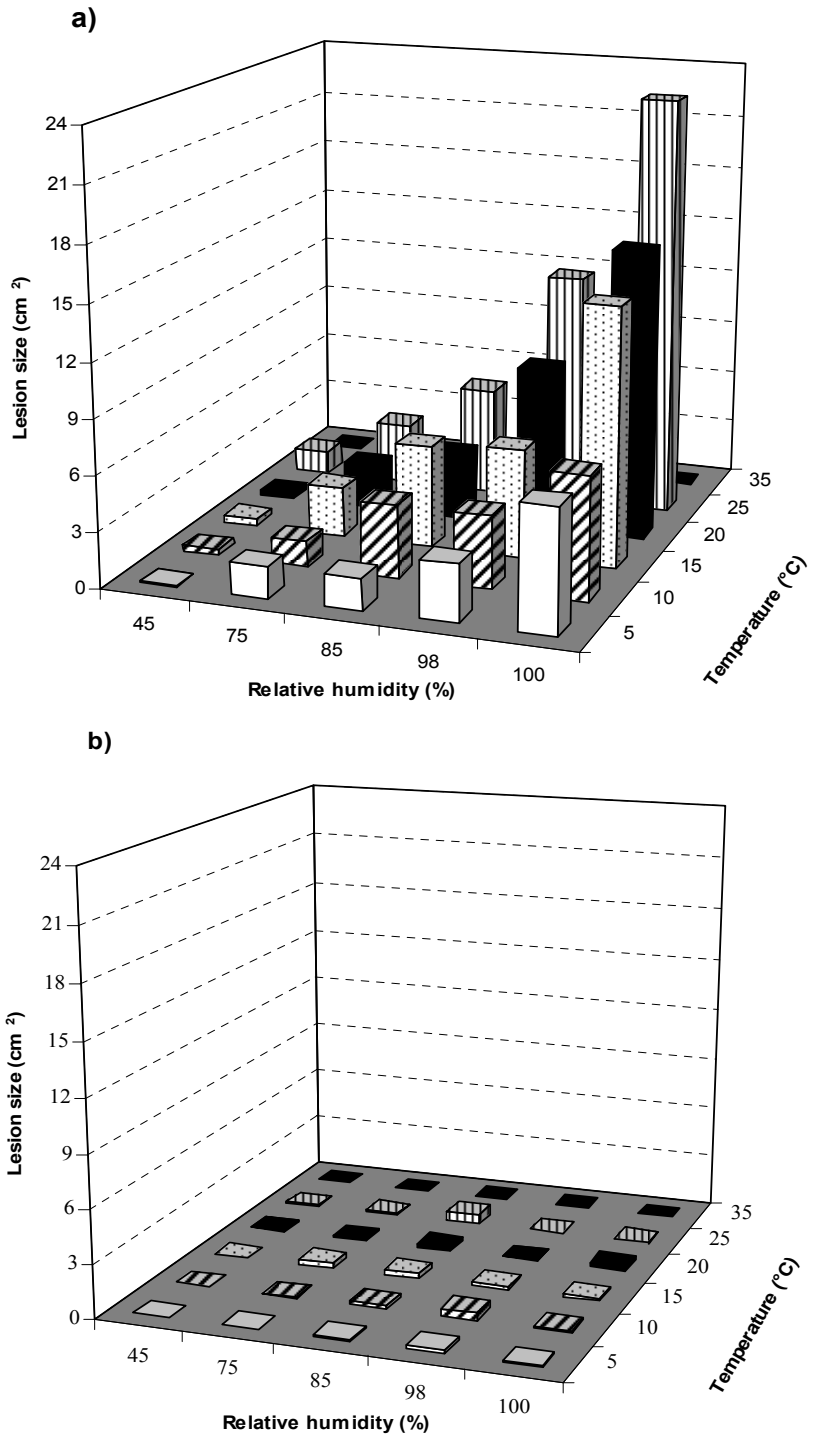

Fig. 2. Effect of temperature and relative humidity on lesion size of *P. italicum* on Valencia late oranges applied alone or 24 h after antagonistic yeast strain Z1 application (b).