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Microcalorimetric measurement of *Trichoderma* spp. growth at different temperatures

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ABSTRACT

The growth of six *Trichoderma* spp. warm-weather strains (WWS) and temperate-weather strains (TWS) was determined by microcalorimetry at different temperatures from 10 to 35 °C. The metabolic heat production (p) and the apparent activation energy (E_a) were calculated. Significant differences among the strains were noted between 20 and 30 °C. The optimal growth temperature was 30 °C for strains TC74, T359, T479, and Th and was 35 °C for TC74M and T397. The p values were from 18 to 28 mW g^{-1} dry weight of mycelium and 11–16 mW g^{-1} of dry weight of mycelium for the WWS and TWS, respectively. The highest and lowest E_a values were observed for T479 (98 kJ mol^{-1}) and Th (34 kJ mol^{-1}), respectively. For WWS, the p value at the highest growth was directly related to antibiosis, and the E_a values were directly correlated to sclerotium parasitism. Both p and E_a were inversely related to sclerotium parasitism and pathogen growth inhibition, respectively. By contrast, the p value for TWS at maximal growth was inversely related to antibiosis while the E_a was directly related. Microcalorimetry was used to detect minute differences in metabolism heat among the *Trichoderma* strains, providing a rapid and accurate way to yield reproducible data.

1. Introduction

Among the microorganisms studied for biological control of plant diseases, fungi of the genus *Trichoderma* have been widely studied. New strains are constantly being evaluated to better understand their biocontrol potential. The selection of microorganisms for biological control initially requires their growth assessment to evaluate their capacity to explore the three-dimensional habitat space or to compete for nutrients and space. *Trichoderma* species have been evaluated through *in vitro* assays where the antagonists grow under specific controlled environments and are exposed to several factors of interest [1–3]. In particular, temperature strongly influences growth and secondary metabolites production of *Trichoderma* species [4–7]. The remarkable physiological similarities among species make it challenging to detect differences in their growth with Petri dish-based assays [7].

Metabolic processes during microbial growth produce heat that can be described by the net heat change [8–13]. These changes can be determined by microcalorimetry [10–16], a very sensitive, non-destructive technique that requires only small quantities of samples to yield fast and reproducible data [3,17,18]. This technique has proven to be sensitive towards changes in the induced microbial biomass, which cannot be detected by conventional methods [19]. Microcalorimetry has been widely used to determine microbial activity in the soil [8,17,20]; in addition, its application has recently been extended to fungal growth studies. Therefore, this method could give qualitative and quantitative information throughout the course of an experiment [15,20,21] for metabolic activity determination (heat rate, p and CO_2 rate, R_{CO_2}), thereby allowing for a mathematical description of the growth rate ($R_{SG} \cdot \Delta H_B$) [17,18].

The present study aimed to describe the use of isothermal microcalorimetry to evaluate the metabolic response of several *Trichoderma* spp. strains under different growth temperatures.

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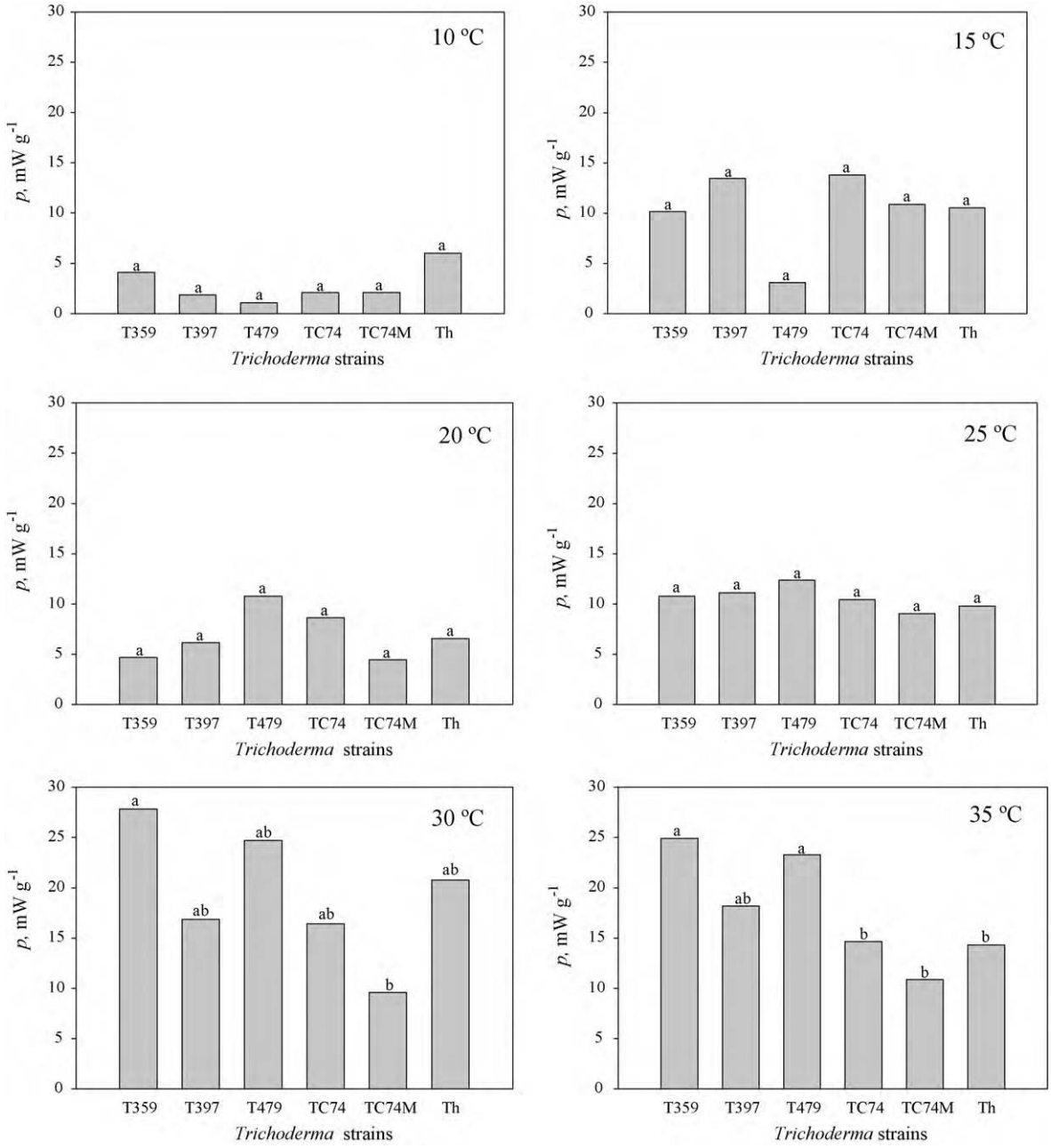

Fig. 1. Metabolic heat (p) of *Trichoderma* strains growth at different temperature. Strains T359, T397 and T479 are warm-weather strains (WWS). Strains TC74 and TC74M are temperate-weather strains (TWS). Th is reference strain.

2. Materials and methods

2.1. Strains

The plant pathogen fungi *Phymatotrichopsis omnivora* was provided by Dr. Jose Antonio Samaniego-Gaxiola from La Laguna Experimental Station, INIFAP, Coahuila, Mexico. *Trichoderma* strains T359, T397 and T479 were sampled from warm-weather strains (WWS) from the State of Guerrero, Mexico (mean temperature of 37 °C) provided by Dr. Alejandro Michel-Aceves (Colegio Superior Agropecuario del Estado de Guerrero, Guerrero, Mexico). The temperate-weather strains (TWS) TC74 and TC74M were sampled from the State of Chihuahua, Mexico (mean temperature of 27.9 °C). These strains were selected due to their remarkable antagonistic effect against pathogens, such as *Phytophthora capsici*, *Rhizoctonia solani*, *Macrophomina phaseolina* and *Sclerotium rolfsii* [22–24]. The *Trichoderma harzianum* strain (Th) is commercially available (ProSelective™ Naturalmente Pureza, S.A. de C.V. Durango, Mexico) and was used as a reference.

2.2. Trichoderma spp. and P. omnivora growth kinetics in PDA plates

Every strain was inoculated in the center of a potato dextrose agar (PDA) plate (90 mm of diameter) at 28 °C until the plate surface was fully covered. Radial growth was measured daily, and the data were analyzed as a completely randomized design with four replications using SAS for Windows v. 8. The growth rate (GR) was calculated at the end of the experiment by linear regression. This experiment was performed twice.

2.3. Microcalorimetric measurements

To determine the influence of temperature on growth and metabolic activity, the *Trichoderma* spp. strains were grown in PDA at 28 °C for 24 h; afterwards, 4-mm mycelium discs were cut and incubated for an additional 12–18 h [25]. Two discs of each *Trichoderma* strain sample (average weight 8.0 mg of mycelium) were analyzed with a model CSC 4100 differential scanning calorimeter (DSC, Calorimetry Science Corporation, Pleasant Grove, Utah) with four 1-cm^3 metallic hermetic ampoules, a ±1 μW baseline and a scanning capacity ranging from −10 to 110 °C. Each of the first three ampoules contained two discs of a *Trichoderma* strain sample; the fourth ampoule contained two fungus-free PDA discs as a reference. After each measurement, the DSC ampoules were cleaned with absolute methanol and rinsed with distilled water to avoid cross contamination. Specific thermal power (p) was measured from 10 to 35 °C with 5 °C increments and sample incubation for 50 min in each temperature. The isothermal data were baseline corrected and adjusted to the sample dry weight. The p values were logarithmically transformed to produce an Arrhenius graph [18]; the apparent activation energy for each strain was calculated with a best-fit line according to the following equation: $E_a = \beta \times R$ (8.314 J mol^{-1} K^{-1}), where E_a is the activation energy, β is the slope of the linear equation, and R is the gas constant. Data were analyzed as a completely randomized design with three replications using SAS for Windows v. 8.

2.4. Antagonism assays

The *Trichoderma* antagonistic effect on *P. omnivora* was evaluated by dual culture confrontation, sclerotium parasitism and antibiosis methods. For the dual culture confrontation, one 8-mm discs of PDA, containing *P. omnivora* mycelium, was placed in the perimeter of a PDA Petri dish and incubated at 28 °C for 72 h. Afterward, one similar disc, but containing *Trichoderma* mycelium was placed in opposite edges of the Petri dish and the incubation was extended for 6 days or until the *Trichoderma* mycelium covered all plate. The growth was measured daily, and the growth inhibition (GI) was calculated by considering *P. omnivora* growth in the absence of *Trichoderma* as 100%.

To investigate the influence of *Trichoderma* on the parasitism of sclerotium, a total of 10^6 *Trichoderma* conidia (1 mL) were uniformly distributed on the surface of a 2% agar plate. Five *P. omnivora* sclerotia were added to each plate and incubated for 144 h. Daily observations were performed to detect any sclerotium colonization by the antagonist.

Table 1
Growth rate (GR), time to maximum mycelium growth (t), optimal temperature (T), maximum metabolic heat (p) and apparent activation energy (E_a) for *Trichoderma* spp. strains and *P. omnivora*.

Strain	GR (mm/h)	t (h)	T (°C)	p (mW g^{-1})	E_a (kJ mol^{-1})
TC74	1.19b	67b	30	16ab	39a
TC74M	1.19b	67b	35	11b	54a
T479	1.21b	66b	30	25ab	98a
T397	1.88a	42a	35	18ab	81a
T359	1.84a	42a	30	28a	62a
Th	1.19b	67b	30	21ab	34a
Po	0.51c	157c	–	–	–

Growth on PDA plates at 28 °C. *P. omnivora* (Po) is included. Time to maximum mycelium growth was calculated based on the growth rate and the Petri dish diameter (90 mm). Optimal temperature values according to metabolic heat production. Significantly different data (Tukey test, $P < 0.05$) within the same column are marked with different letters. Strains T359, T397 and T479 are warm-weather strains (WWS). Strains TC74 and TC74M are temperate-weather strains (TWS). Th is the reference strain.

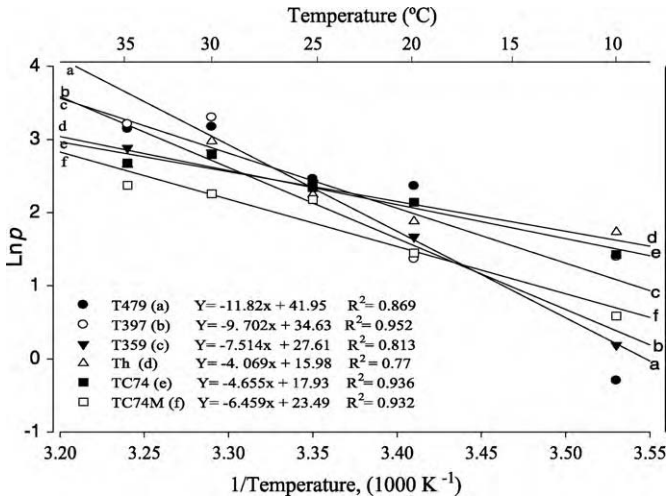

Fig. 2. Arrhenius Graph for growth of *Trichoderma* strains at different temperatures. Strains T359, T397 and T479 are warm-weather strains (WWS). Strains TC74 and TC74M are temperate-weather strains (TWS). Th is reference strain.

For the antibiosis experiments, each PDA plate was covered with a sterile cellophane membrane before placing a *Trichoderma* mycelium disc in the center. The plates were incubated at 30 ± 2 °C for 43 h, and the membrane with the fungus was removed. Control plates were similarly treated but without the antagonist fungus. Afterwards, the plates were inoculated with the pathogen discs, and their growth was measured daily.

All assays were performed in duplicate at 28 °C, and the data were analyzed by means of a completely randomized design with five replications using SAS for Windows v. 8. The percentage values were arcsine transformed before their analysis.

3. Results and discussion

Biocontrol is a useful strategy for controlling soil-borne plant pathogens, and many strains from *Trichoderma* species have been proposed for this use. However, differences of physiological behavior or biochemical capability are expected and can be reflected in the mycoparasitic characteristics of each strain. The radial growth rate is a good parameter for determining the ability of the strain to colonize the substrate and for describing its antagonistic capability. Fungal growth rate is highly dependent on temperature. The differences in metabolic activity can be determined with microcalorimetry by growing the fungus at different temperatures. In this study, the growth of six *Trichoderma* strains was analyzed by microcalorimetry, and the relationship between the calorimetric data and the potential antagonism of the strains was investigated.

3.1. Trichoderma spp. and P. omnivora growth rate

All *Trichoderma* strains grew faster than *P. omnivora* ($P < 0.05$) on PDA medium at 28 °C, as shown in Table 1. Each *Trichoderma* strain had a different growth rate (GR). The warm-weather strains (WWS) T359 and T397 showed faster growth than the temperate-weather strains (TWS) during the first 48 h ($P < 0.05$), but afterwards the growth was similar ($P > 0.05$) for all strains. Due to their faster initial growth, strains T359 (1.84 mm/h) and T397 (1.88 mm/h) achieved their maximum mycelium growth 25 h earlier ($P < 0.05$) than TC74M (1.19 mm/h), TC74 (1.19 mm/h), and the reference strain, Th (1.19 mm/h). In addition, all *Trichoderma* strains showed higher GRs than *P. omnivora* (0.51 mm/h) ($P < 0.05$), covering the surface medium in 42–67 h rather than the 157 h required by the pathogen.

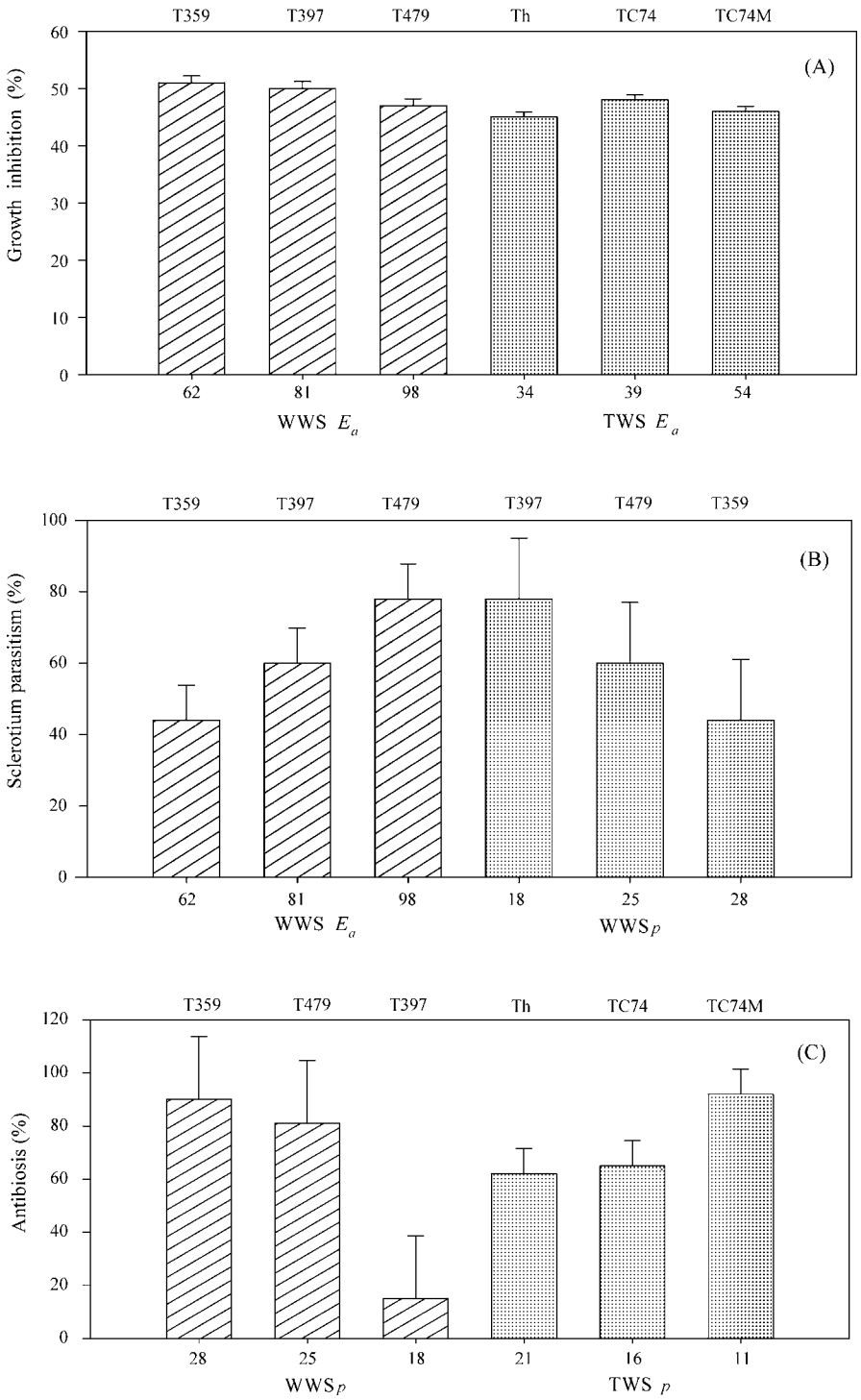

Fig. 3. Microcalorimetric variables and antagonistic relationship. (A) E_a and *P. omnivora* growth inhibition. (B) WWS E_a and p as well as sclerotium parasitism; (C) p and antibiosis. Bars represent the standard deviation.

3.2. Microcalorimetric measurements

Since significant differences were observed among the growth rates of the *Trichoderma* strains, the influence of the incubation temperature on the p values and the E_a required for growth was determined by microcalorimetry (Figs. 1 and 2). All strains generated the same p values ($P > 0.05$) from 10 to 25 °C, but significant differences were noted at 30 and 35 °C (Fig. 1). At 30 °C, strain T359 had the highest p value and TC74M had the lowest (Tukey $P < 0.05$). The other strains had intermediate values. At 35 °C, T359 and T479 (WWS) generated higher p values than any TWS strain (Tukey $P < 0.05$). At 30 °C, T359 and T479 showed the greatest p values (28 and 25 mW g^{-1} of dry mycelium, respectively). The maximum p value for TC74 was 16 mW g^{-1} of dry mycelium. The maximum p value for TC74M was 11 mW g^{-1} of dry mycelium at 35 °C and was the lowest obtained p value. The Th strain generated a maximum of 21 mW g^{-1} of dry mycelium. The p curves generated by each *Trichoderma* strain had a close relationship with their growth rate. These results are similar to those reported in other studies [18–20].

Table 2
Variables related to antagonism of *Trichoderma* strains. Growth rate (PGR) and growth inhibition (PGI) of *P. omnivora*, *Trichoderma* overgrowth over mycelium of *P. omnivora* (TO), sclerotium parasitism (SP) and antibiosis (A).

Strain	PGR (mm/h)	PGI (%)	TO (%)	SP (%)	A (%)
TC74	0.31ab	48a	100a	100a	65a
TC74M	0.40a	46a	100a	0d	92a
T479	0.34ab	47a	100a	78ab	81a
T397	0.24b	50a	100a	60bc	15b
T359	0.29ab	51a	100a	44bc	90a
Th	0.35ab	45a	100a	96a	62a

Growth was monitored on PDA plates at 28 °C, except for sclerotium studies, which were done on water agar plates. The *P. omnivora* growth rate (0.5052 mm/h), when grown without an antagonist, was statistically different (Tukey $P < 0.05$). Significantly different data (Tukey $P < 0.05$) within the same column are marked with different letters. Strains T359, T397 and T479 are warm-weather strains (WWS). Strains TC74 and TC74M are temperate-weather strains (TWS). Th is the reference strain.

The effect of temperature on p can be seen in the Arrhenius graph (Fig. 2), where TC74M, T397 and T479 had higher slope values than those of TC74, T359 and Th. The highest linearity of curves was between the 3.4 and the 3.3 levels. The E_a values in this interval (3.41–3.29 1000 K^{-1}), which corresponds to 20–30 °C, fluctuated from 34 to 98 kJ mol^{-1} (Table 1). The greatest value was for strain T479, followed by T397, while strain Th had the lowest E_a. Even though there were not significant differences, the WWS had greater E_a values than those of the TWS. Furthermore, as shown in the Arrhenius graph, the p values increased with temperature until 30 °C; at this point, decreasing values were observed, except for the TC74M and T397 strains. According to the Arrhenius equation, p exponentially increases with incubation temperature up to the optimal growth temperature. Therefore, the optimal growth temperature was 30 °C (3.29 1000 K^{-1}) for most strains, except for TC74M and T397, which grew optimally at 35 °C (3.24 1000 K^{-1}). Several authors [1,7,26–28] have reported that the optimal growth temperature for *Trichoderma* spp. is 30 °C, although some strains grow very well in the range of 25–30 °C and some species, such as *T. longibrachiatum*, can grow at 40 °C [29].

3.3. Antagonism assays

All *Trichoderma* strains showed antagonistic activity against *P. omnivora.* The GR of the pathogen was significantly reduced in the presence of any *Trichoderma* strain ($P < 0.01$) and the growth of *P. omnivora* stopped after contact with the antagonist in all cases (Table 2). The final overgrowth level was similar for all *Trichoderma* strains; T359 and T397 overgrew *P. omnivora* in 96 h while the other strains required at least 114 h. In contrast, significant differences ($P < 0.01$) among the strains were observed in their sclerotium parasitism and antibiosis properties (Table 2). All strains, with the exception of TC74M, parasitized sclerotium; TC74 showed the highest parasitic activity (Tukey $P < 0.05$), followed by Th, while T359 had the lowest antagonistic activity. Differences in antibiosis activity were observed among the *Trichoderma* strains, although only T397 was statistically significant (Tukey $P < 0.05$) and showed a discrete activity. All of the other strains were capable of inhibiting the parasite growth from 62 to 92%.

When the strains were analyzed as a group, a significant correlation could not be established between the calorimetric data and any antagonistic activity. However, when the strains were grouped by origin, a correlation was observed (Fig. 3). For the WWS strains, the E_a was inversely associated to pathogen growth inhibition ($R^2 = 0.918$) (Fig. 3A) and directly related to sclerotium parasitism ($R^2 = 0.998$) (Fig. 3B). Similarly, the p and antibiosis capability of the WWS strains exhibited a direct relationship ($R^2 = 0.965$) (Fig. 3C). However, p was inversely related with sclerotium parasitism (Fig. 3B). For the TWS strains E_a was directly related to antibiosis ($R^2 = 0.978$) while p and antibiosis had an inverse relationship ($R^2 = 0.824$) (Fig. 3C).

Our results showed that the antagonistic activities of the evaluated strains are temperature dependent and that WWS and TWS have different thermal requirements for biocontrol processes. The WWS strains mainly produce antibiosis factors, while the TWS strains work better as sclerotium parasites. The behavior of WWS is compatible with one WWS, *T. longibrachiatum*, which was reported to be an excellent antibiotic producer but with limited mycoparasitic activity [30]. On the other hand, the temperate-weather strains, but not the warm-weather strains, seem to have different thermal requirements for growth and for the production of secondary metabolites. These results are similar to those previously reported for other mesophylic fungi [6,31].

The TC74 strain had the same growth rate but lower energy requirements than those of the WWS strains; however, its antagonistic activity was less affected by the incubation temperature. This strain exhibited mycelium growth inhibition and was most effective against sclerotium. These results indicate that TC74 could be an efficient antagonist agent for the control of *P. omnivora.* In addition, TC74 has activity over a wider range of temperature. Therefore, TC74 has the key characteristics required for its use on soils from arid environments, where fluctuating temperatures and *P. omnivora* are common.

4. Conclusions

Microcalorimetry is a sensible and reproducible tool that is capable of detecting minute differences in metabolic heat among *Trichoderma* strains. Therefore, the influence of incubation temperature on growth was determined by a rapid and accurate way. Different incubation temperatures could be tested in one experiment in a short time (50 h) and reproducibly. In addition, the growth conditions could be controlled, producing similar environmental conditions during the whole experiment.

Correlations between the calorimetric parameters and antagonistic activity were not found when the *Trichoderma* strains were considered as a group. However, when the strains were grouped based on their origin (warm or temperate weather), a correlation was established. Our results also revealed the effect of temperature on the metabolic activity of the *Trichoderma* spp. strains; the most significant effects were observed between 20 and 30 °C. In most cases, the optimum growth temperature of the strains was 30 °C. The antagonistic activity of the *Trichoderma* strains was temperature dependent, especially for the WWS strains, whose biocontrol capacity was affected by their activation energy. The WWS and TWS strains have different thermal requirements for biocontrol. The WWS strain seems to work better as an antibiosis producer, while sclerotium parasitism is the main antagonistic activity of the TWS.

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