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Effects of Temperature on Growth and Siderophore Production of *Pseudomonas Fluorescens-Putida* Strains*

B. DIGAT and J. MATTAR

INRA–Station de Pathologie végétale et Phytobactériologie 49000 Angers, France.

Abstract

Experiments performed at 25°C and 35°C on growth *in vitro* of fifty strains of the *Pseudomonas fluorescens-putida* cluster originating from temperate and tropical countries, allowed the establishment of an ecological classing according to the relative thermophilic nature of the strains. All the strains which grew normally at 35°C were from tropical origin. Indeed at this temperature, most of the strains from temperate countries grew slowly or not at all and they did not produce any fluorescent siderophores with a few exceptions, while tropical strains produced siderophores. Moreover it was observed that exopolysaccharide (EPS) synthesis is wholly inhibited at 35°C for all the strains (temperate and tropical strains as well). When the bacterial strains are subjected to a temperature of 41°C for 48 hours, resistance to this thermal stress is generally correlated to the tropical origin of the strain. Finally the colonizing behaviour of the *Pseudomonas fluorescens-putida* strains in relation to rhizosphere and tomato root systems differs on a characteristic way in terms of temperature. Assessing tomato roots colonizing capacity of a temperate (L26-1) and a tropical (M3-1) strains, it was observed that the tropical strain had a better colonizing capacity at 21°C and 33°C as well. Consequences of this thermosensitivity on establishment, survival and development of PGPR populations in substrates and in soils are discussed.

1. Introduction

Among environmental effects influencing the interactions between Plant-Growth-Promoting Rhizobacteria (PGPR) and rhizosphere, temperature is one of the key factors. The *Pseudomonas fluorescens-putida* group is characterized by a great variability of strains (Digat and Gardan 1987) that can be isolated worldwide. Generally strains of various temperate and tropical biotopes have an optimum growth temperature between 25°C and 30°C. However, above 30°C, physiological functions of certain strains are affected. For instance, synthesis of siderophores or fluorescent pigment of

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some fluorescent *Pseudomonas* is inhibited above 33°C (Loper and Schroth 1986).

Temperatures above 33°C are frequently observed during several hours in greenhouse conditions, in soil-less culture, in the superficial stratums of soils in temperate and tropical countries. These high temperatures may affect microflora populations *in situ*. High soil temperatures can influence the fate of rhizosphere population of introduced fluorescent *Pseudomonas* (Loper et al., 1985). Therefore, the investigation of the influence of temperature on the "*in vitro*" and "*in situ*" behaviour of *P. fluorescens-putida* group is of microbiological interest for strain characterization and of ecological interest for the selection of strains able to offer resistance to temperature stress in soils and in substrates and to understand better the biology of soil *Pseudomonas*.

2. Material and Methods

Bacterial strains

The purified strains were identified as belonging to *Pseudomonas fluorescens-putida* group according the classic scheme (Stanier et al., 1966). Fifty strains were used in this study. They were isolated from various host plants rhizosphere originating from several countries in the world. Only seventeen representative strains from various geographical origins were selected for the study of the resistance to temperature stress (see Table 1).

Growth and Production of fluorescent pigment in vitro *at 25°C and 35°C*

On King's medium B (King et al., 1954) Agar plates

Inoculation of King's medium B agar plates was performed using a bacterial suspension of 10^5 c.f.u./ml made from a 48 hour old culture. The colonies were observed after 24, 48 and 72 hours of incubation. Growth and extracellular polysaccharide (EPS) production were observed visually and compared between them after 48 hours. The fluorescent pigment production of bacterial colonies was observed at 365 mm with a UV lamp. Two successive experiments including two replications for each strain were performed.

On Misaghi's medium (Misaghi et al., 1982)

For each strain, the inoculum was made from a bacterial suspension of 10^8 c.f.u./ml precisely adjusted by turbidity. Fifty µl of this suspension were mixed to 6 ml of Misaghi medium in glass tube (Ø 16 mm) and shaken at 140 rpm Growth was determined by turbidity every 12 hours during four days. Three replications for each strain were used.

Temperature stress resistance

The inoculum was prepared as above in Misaghi's medium in order to obtain a final

Table 1. Influence of temperature on growth, fluorescence and resistance to thermal stress of *P. fluorescens-putida* strains isolated from France and Sicily

Strain	Host-plant	Country of Origin	25°C		35°C		41°C-48h
			Growth*	Fluo**	Growth*	Fluo**	*** Resistance
C3.1	Carrot	France	+	+	(−)	−	
C8	Carrot	France	+	+	(−)	−	
C33.1	Carrot	France	+	+	(−)	−	
C25.3	Carrot	France	+	+	−	−	
C27.2	Carrot	France	+	+	−	−	
CSC1	Rape	France	+	+	−	−	
CGV6	Rape	France	+	+	−	−	
Ch22	Chicory	France	+	+	+	−	−
Ch4	Chicory	France	+	+	−	−	−
EPB	Spinach	France	+	+	+	−	
EPC3	Spinach	France	+	+	(−)	−	−
EPD	Spinach	France	+	+	(−)	−	
EPF	Spinach	France	+	+	−	−	−
EPH3	Spinach	France	+	+	+	−	
G92	Apple tree	France	+	+	+	−	
H6	Sugar beet	France	+	+	+	+	−
LSG1	Sugar beet	France	+	+	+	−	
LSG2	Sugar beet	France	+	+	+	+	
NSF5	Sugar beet	France	+	+	+	−	
L26.1	Lettuce	France	+	+	+	−	−
Pe3	Pansy	France	+	+	−	−	−
Pe4	Pansy	France	+	+	+	−	
Pe11	Pansy	France	+	+	+	−	
Pois A1	Pea	France	+	+	(−)	−	
Pois A3	Pea	France	+	+	(−)	−	−
Pi D	Red pepper	Sicily	+	+	+	+	−
Ti A2	Tomato	Sicily	+	+	+	−	
Ti a2	Tomato	Sicily	+	+	+	+	
Ti b32	Tomato	Sicily	+	+	+	+	
T21	Tomato	Sicily	+	+	+	−	+

* + or (−) or −: Visible growth, very weak growth or no growth respectively on King's medium B
** + or −: Fluorescence or no on King's medium B
*** + or −: Resistance or no in Misaghi's medium

concentration of 10^5 c.f.u./ml. The incubation was performed in that medium at 41°C for 0 (control), 12, 24 and 48 hours. Then, glass tubes were shaken at 26°C at 125 rpm for 96 hours. Growth was determined by turbidity after 24, 48, 72 and 96 hours. Three replications for each strain were performed.

Bacterial growth at 21° and 33°C in tomato rhizosphere

Plant material and plant cultivation

Tomato cv. "Tresor" seeds were disinfected by dipping them in calcium hypochlorite

5% solution for 7 minutes and rinsing in sterile water. Disinfected seeds were sown directly on sterile rockwool cylinders (100 × 20 mm) in sterile glass tubes (150 × 22,5 mm) and sealed with plastic caps. Cultivation conditions were: ambient temperature (25°C day/21°C night); substrate temperature stabilized by thermostated waterbaths at 21°C and 33°C; light intensity of 10 000 lux with a photoperiod 12 hours day/12 hours night; water supply with a nutrient solution at pH 5.8. Five days after sowing in tubes, seedlings were distributed according to the thermal treatments in waterbaths.

Bacterial strains and method of inoculation

Two strains of *Pseudomonas fluorescens-putida* cluster were used: a "temperate" strain L26-1 isolated from lettuce rhizosphere (France) and a tropical strain M3-1 isolated from maize rhizosphere (Thailand). These strains were selected because their capacity of *in vitro* antagonism against *Pythium spp, Phytophthora capsici, Rhizoctonia solani, Sclerotinia minor, Fusarium oxysporium.* The bacterial strains were grown with shaking (160 rpm) in Misaghi's medium. Every rockwool cylinder was inoculated by a bacterial suspension diluted in the nutrient solution so as to obtain 10^7 c.f.u/g rockwool.

Dynamic of bacterial population in the substrate and tomato root colonization

Four bacterial counts were achieved on 4, 7, 14 and 21 days after sowing. Cylinders of substrate were opened and vigorously shaken in 150 ml of sterile water. Pieces of roots 6 to 30 centimeters long were shaken in 4.5 ml of sterile water. Then *P. fluorescens-putida* L26-1 colonies were identified and counted by dilution method on KB medium followed by sero-agglutination using antiserum made from glycoprotein antigens (Digat and Cambra 1976).

3. Results

Growth and production of fluorescent pigment at 25° and 35°C (see Tables 1 and 2)

On KING's medium B agar plates, all the temperate and tropical strains grew normally and were fluorescent at 25°C. On the contrary, at 35°C, most of the temperate strains grew weakly or did not grow at all, and there was no production of fluorescent pigment but five strains. On this medium, production of extracellular polysaccharide (EPS) was normal at 25°C but was inhibited at 35°C even for the tropical strains.

In the MISAGHI's medium, the temperate strains usually grew and produced fluorescent pigment at 25°C. On the contrary at 35°C, the tropical strains showed a growth curve near that obtained at 25°C (see Fig. 1). At 35°C, the growth of the temperate strains was strongly reduced or did not occur. Some temperate strains which grew weakly on King's medium B agar plates did not grow in the Misaghi's medium.

Table 2. Influence of temperature on growth, fluorescence and resistance to thermal stress of *P. fluorescens-putida* strains isolated from several tropical countries

Strain	Host-plant	Country of Origin	25°C		35°C		41°C-48h
			Growth*	Fluo**	Growth*	Fluo**	*** Resistance
Aub3	Egg-plant	La Réunion	+	+	+	+	+
Aub4	Egg-plant	La Réunion	+	+	+	+	
M2	Maize	Thailand	+	+	+	+	
M3.1	Maize	Thailand	+	+	+	+	+
M3.2	Maize	Thailand	+	+	+	+	
M4	Maize	Thailand	+	+	+	+	
M7.2	Maize	Thailand	+	+	+	−	+
M9	Maize	Thailand	+	+	+	+	
Pt1	Potato	Vietnam	+	+	+	−	
Pt3	Potato	Vietnam	+	+	+	+	+
SL3	Solanum torvum	La Réunion	+	+	+	+	+
SL5	Solanum torvum	La Réunion	+	+	+	+	
SO11	Soya	Thailand	+	+	+	+	+
SO23	Soya	Thailand	+	+	+	+	
SO31	Soya	Thailand	+	+	+	+	
SO32	Soya	Thailand	+	+	+	+	
T112	Tomato	La Réunion	+	+	+	+	+
T211	Tomato	La Réunion	+	+	+	+	
T212	Tomato	La Réunion	+	+	+	+	
T114	Tomato	La Réunion	+	+	+	+	

* + or (−) or −: Visible growth, very weak growth or no growth respectively on King's medium B
** + or −: Fluorescence or no on King's medium B
*** + or −: Resistance or no in Misaghi's medium

Resistance to temperature stress

Results shown in Tables 1 and 2 demonstrate the growth of strains in Misaghi's medium subjected to 41°C for 48 hours. A direct correlation between the geographical origin of strains and their temperature stress resistance was observed. Hence, while the eight strains originating from France were not resistant to this temperature stress, the seven tested tropical strains were resistant and their growth was not affected by the thermal treatment. However, if subjected to 41°C for 12 hours, the tested temperate strains could recover their growth with a delayed exponential phase.

Behaviour of bacterial strains "in situ" *at 21° and 33°C in the substrate and at the tomato root level*

At 21°C, when there was only one strain in the substrate, the temperate strain L26-1 dropped from 10^6 to 10^4 c.f.u. per gram of substrate 21 days after bacterization while at 33°C it dropped only from one log unit. However, at 21°C, the tropical strain M3-1 gained one log unit and at 33°C it gained again one half log unit. When the two strains

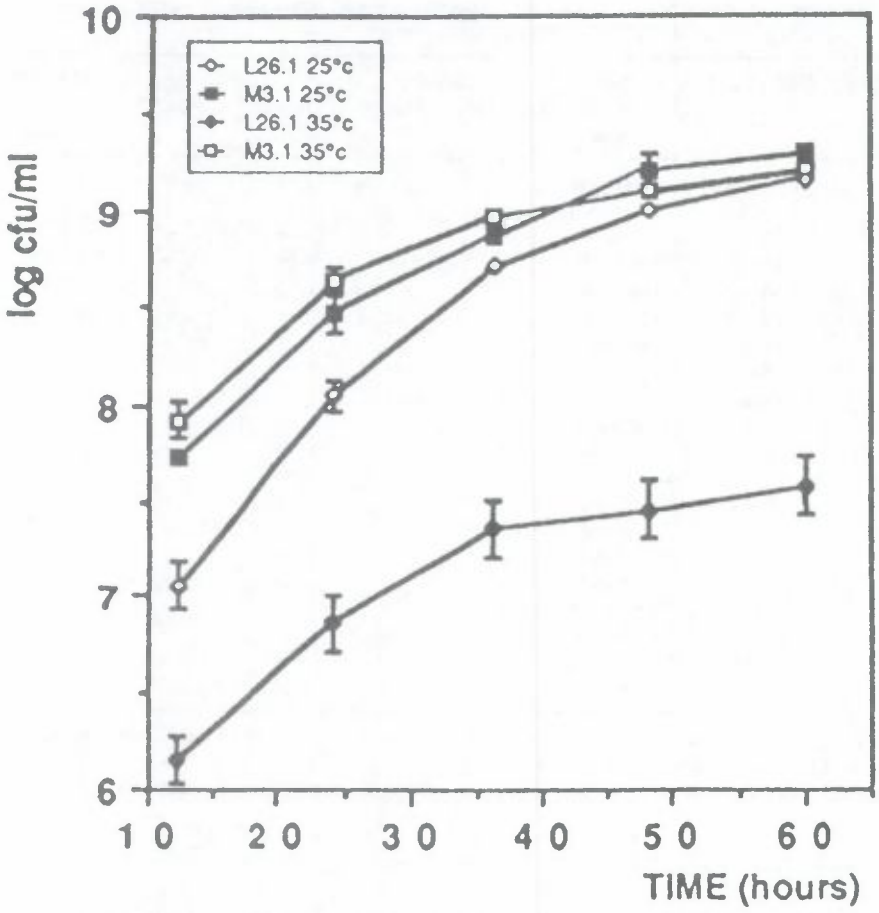

Figure 1. Influence of temperature on growth of *Pseudomonas fluorescens-putida* strains. Temperate strain L26.1 and tropical strain M 3.1 were grown in the Misaghi's medium at 25°C and 35°C. Note the growth of the temperate strain at 35°C which was strongly reduced.

were mixed in the substrate, the strain L26-1 was inclined to disappear at 21°C as well as at 33°C. At the root level, at 21°C and at 33°C, L26-1 population was inclined to disappear 21 days after bacterization; however on the opposite, strain M3-1 maintained a constant population. When the two strains were mixed, strain M3-1 was constant at these two temperatures while strain L26-1 disappeared.

4. Discussion

From the results "*in vitro*", a direct correlation can be established between the geographical origin of the bacterial strains and their behaviour at different temperatures. While the tropical strains grew normally and produced siderophores at 35°C, but two,

the temperate strains did not. Moreover, all the tested tropical strains resisted to a thermal stress of 41°C for 48 hours while the temperate strains were sensitive, but one from Sicily. "*In situ*", in the substrate as on the tomato roots, tropical strain M3-1 maintained populations at 21°C and at 33°C, while temperate strain L26-1 was inclined to disappear in both cases. At 35°C the siderophore synthesis of the temperate strains is highly reduced, while the tropical strains are generally unaffected. Therefore it is speculated that tropical populations of *P. fluorescens-putida* are probably favoured at high temperatures in field conditions because they can obtain ferric cations needed for their energetic metabolism. Consequently, in the case of bacterization of soils or substrates, the balance of bacterial populations could be altered in favour of "warm" strains when the soil temperature is above 30°C. This thermosensitivity of *P. fluorescens-putida* strains should be considered for producing microbial soil inoculants. It seems judicious to make inoculants from a mixture of strains having a complementary thermosensitivity.

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