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Influence of temperature on plant–rhizobacteria interactions related to biocontrol potential for suppression of fusarium wilt of chickpea

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Seed and soil treatment with *Pseudomonas fluorescens* RGAF 19, *P. fluorescens* RG 26, *Bacillus megaterium* RGAF 51 and *Paenibacillus macerans* RGAF 101 can suppress fusarium wilt of chickpea (*Cicer arietinum*), but the extent of disease suppression by these rhizobacteria is modulated by soil temperature. In this work, the effect of temperature on plant–rhizobacteria interactions was assessed in relation to biocontrol potential for suppression of fusarium wilt of chickpea. Seed and soil treatment with those rhizobacteria delayed seedling emergence compared with nontreated controls, and either increased or had no deleterious effect on chickpea growth. *Pseudomonas fluorescens* isolates significantly increased chickpea shoot dry weight at 20°C and root dry weight at 25 and 30°C. All bacterial isolates colonized the chickpea rhizosphere and internal stem tissues at 20, 25 and 30°C, and there was a positive linear trend between bacterial population size in the rhizosphere and temperature increase. The maximum inhibition of mycelial growth and conidial germination of *Fusarium oxysporum* f. sp. *ciceris* race 5 *in vitro* occurred at a temperature range optimal for bacterial growth and production of inhibitory metabolites. These results demonstrate the need to understand the effects of environmental factors on the biological activities of introduced rhizobacteria of significant importance for plant disease suppression.

Keywords: antibiosis, chickpea, endophytes, *Fusarium oxysporum* f. sp. *ciceris*, plant growth promotion, pyoverdine

Introduction

The use of bacteria as biocontrol agents of soilborne plant pathogens, as an alternative or complementary strategy to physical and chemical disease management, has been investigated for over 70 years (Weller, 1988). The lack of consistency in performance of beneficial bacteria such as *Pseudomonas* spp. or *Bacillus* spp. under field conditions has limited their use in commercial agriculture (Raaijmakers *et al*., 2002). Much of that inconsistency has been attributed to variability in physical and chemical properties within niches occupied by biocontrol agents that affect both colonization and expression of biocontrol mechanisms such as antibiosis, parasitism, competition and induced resistance (Ownley *et al*., 2003).

Many abiotic soil factors, such as pH, temperature, moisture, texture, and inorganic and organic constituents, may influence the mechanisms of biocontrol. There is, however, little knowledge of how specific factors affect the interactions among soilborne plant pathogens and their antagonists (Burpee, 1990; Duffy & Défago, 1999; Ownley *et al*., 2003). Factors that influence the dynamics of antagonist populations may not necessarily affect mechanisms of antagonism in the same way. In fact, the specific activities occurring during antagonistic growth, such as antibiotic production, induction of host defence responses, etc., are probably more important in disease suppression than increase of the antagonist population in the rhizosphere (Lewis & Papavizas, 1985).

Temperature is a key factor influencing both colonization by rhizobacteria and expression of biocontrol mechanisms (Burpee, 1990; Beauchamp *et al*., 1991). Soil temperature influences biological control by: (i) affecting the natural disease suppressiveness of soils (Olsen & Baker, 1968); (ii) predisposing pathogens to microbial antagonism (Munnecke *et al*., 1976; Henis & Papavizas, 1983); (iii) regulating the growth or production of metabolites (such as antibiotics and siderophores) by specific antagonists (Adams & Ayers, 1980; Loper & Schroth,

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1986; Paulitz & Baker, 1987; Raaijmakers *et al*., 2002), or (iv) modulating disease development and consequently the level of disease suppression achieved (Landa *et al*., 2001).

In recent work, it was demonstrated that incubation temperature and inoculum density of *Fusarium oxysporum* f. sp. *ciceris* race 5 strongly interacted in modulating the suppression of fusarium wilt in chickpea (*Cicer arietinum*) by four rhizobacteria: *Pseudomonas fluorescens* RGAF 19, *P. fluorescens* RG 26, *Bacillus megaterium* RGAF 51 and *Paenibacillus macerans* RGAF 101 (Landa *et al*., 2001). Disease suppression by rhizobacteria decreased as conditions became more favourable for disease development. However, the mechanism by which temperature affects suppression was not explored.

The research presented here investigated interactions of temperature with activities of rhizobacteria in relation to the efficiency of their suppression of fusarium wilt. The specific objectives of the study were: (i) to determine the influence of temperature on rhizosphere and stem colonization by *P. fluorescens* strains RGAF 19 and RG 26, *B. megaterium* RGAF 51 and *P. macerans* RGAF 101; (ii) to determine the effect of temperature on the emergence and growth of chickpea inoculated with the rhizobacteria; and (iii) to determine the influence of incubation temperature on *in vitro* antagonism between *F. oxysporum* f. sp. *ciceris* race 5 and the rhizobacteria.

Materials and methods

Bacterial and fungal isolates, and inoculum production

The monoconidial isolate Foc 8012 of *F. oxysporum* f. sp. *ciceris* race 5 used in these *in vitro* studies was employed in previous research (Landa *et al*., 1997, 2001; Navas-Cortés *et al*., 1998). Conidial suspensions were obtained as described in Landa *et al*. (2001). The concentration was adjusted to approximately 10^3 conidia mL^{-1}; suspensions were stored at 4°C and used within 3 h.

Four bacteria effective in suppressing fusarium wilt (Landa *et al*., 1997, 2001) were used in this study. Bacterial cell suspensions were obtained by pipetting 5 mL of sterile 0·1 M $MgSO_4$ onto slants of King's B agar (KBA) (King *et al*., 1954) (*P. fluorescens* RGAF 19 and RG 26) or potato dextrose agar (PDA) slants (*B. megaterium* RGAF 51 and *P. macerans* RGAF 101) and gently shaking to wash cells from the agar surface. Concentrations of bacteria in the suspensions were determined by measuring absorbance at 600 nm (A600) using standard curves for each bacterial isolate.

Effect of incubation temperature on chickpea seedling emergence, growth promotion, and rhizosphere and stem colonization by rhizobacteria

Experiments were conducted in a model chickpea system (Landa *et al*., 2001) using a heat-treated soil mixture. All experiments were conducted in three Fitotron™ (Sanyo Gallencamp PLC, Leicester, UK) growth chambers adjusted to each of 20 ± 1, 25 ± 1 and 30 ± 1°C, with 60 ± 5%/90 ± 5% day/night relative humidity and a 14-h photoperiod of fluorescent light at 360 μE m^{-2} s^{-1}.

Bacterial seed and soil treatment

Chickpea cv. PV 61 ('kabuli' type), susceptible to *F. oxysporum* f. sp. *ciceris* race 5 (Landa *et al*., 2001), was used in all experiments. Treatment of both seed and soil with bacteria, control treatments and estimation of bacterial inocula on the treated seeds and in the soil were conducted as described by Landa *et al*. (2001). Bacteria were applied to reach an inoculum density of approximately 10^8 colony-forming units (cfu) per seed and 10^6 cfu/g soil. Treated seeds yielded $0{\cdot}5 \times 10^8$–$2{\cdot}0 \times 10^8$ cfu per seed, and bacterial inoculum density in soil ranged from $9{\cdot}2 \times 10^5$–$2{\cdot}5 \times 10^6$ cfu/g soil, depending on the bacterial isolate and experiment.

Effect of temperature and bacterial treatment on chickpea emergence

Chickpea seeds treated with a bacterial isolate were sown (48 seeds; eight rows × six seeds per row) in sterile trays (60 × 40 × 10 cm) filled with a soil mixture (clay loam/peat, 2:1, v/v) either autoclaved (121°C for 1 h, twice, on two consecutive days) or pasteurized (70°C for 30 min), and infested with the same bacterial isolate. There were two trays for each experimental combination. The number of seedlings emerging in each tray were recorded every 12 h from the time of sowing for 6 days, and seedlings in the trays were watered as needed. Seedling emergence increase curves (EICs) were obtained from accumulated seedling emergence (E), and used to determine the following variables: (i) time to emergence (t_e) estimated as time in hours for $E > 0\%$; (ii) hours to reach $E = 50\%$ ($t_{50\%}$); (iii) final percentage emergence (FE); and (iv) standardized area under the EICs (SAUEIC) calculated by the trapezoidal integration method and standardized by the duration of the experiment in hours (Campbell & Madden, 1990).

Effect of temperature on chickpea growth and rhizosphere and stem colonization by bacteria

Chickpea seeds treated with a bacterial isolate were sown in 15-cm-diameter clay pots filled with a pasteurized soil mixture infested with the same isolate. Plants were grown in the growth chamber as described above for 45 days, watered as needed and fertilized with 100 mL of 0·1% hydro-sol fertilizer (Haifa Chemicals, Ltd, Haifa, Israel; 20-5-32, N-P-K + micronutrients) solution every week. There were eight replicate pots with one plant per pot. At the end of the experiment, shoots and roots were severed at the point of seed attachment. Shoot length and fresh and dry shoot and root weights were then determined. Roots were washed free of soil, and shoots and roots were dried in an oven at 70°C for 3 days.

The ability of bacteria to colonize internal chickpea tissue was determined in a similar experiment. Stems were rinsed for 5 min under running tap water, disinfested in 0·2% NaOCl for 5 min, and cut into pieces 5–10 mm

long. Three fragments from each of the first/second (low), fourth/fifth (medium) and ninth/10th (high) chickpea internodes were placed onto salt-V8-agar *Bacillus*-semiselective medium (BSV8A) for *B. megaterium* RGAF 51 and *P. macerans* RGAF 101 isolates (Turner & Backman, 1991), or onto KBA supplemented with ampicillin (40 μg mL^{-1}), chloramphenicol (13 μg mL^{-1}) and cycloheximide (100 μg mL^{-1}) (KBA^{+++}) (Simon & Ridge, 1974) for *P. fluorescens* isolates, and incubated at 28°C for 3–4 days. Endophytic colonization was recorded when bacteria grew from internal chickpea tissue at the cut ends of stem pieces. There were eight replicate pots with four plants per pot.

Rhizosphere colonization by bacteria, as influenced by soil temperature, was studied using either bacteria-treated seed or both treated seed and soil as described above. Chickpea seeds treated with a bacterial isolate were sown in conical plastic tubes (175 mm long, 56 mm top diameter; Super-Leach Container, Bardi, Peralta, Spain) (one seed per tube) filled with the autoclaved soil mixture infested or not with the same bacterial isolate. Plants were grown for 10 days in the growth chambers as described above. Plants were then carefully removed from the tubes and their roots were gently shaken to remove all but the most tightly adhering rhizosphere soil and cut into 1 cm segments. The segments from two plants in a treatment were pooled. Three subsamples of 1 g root tissue were placed into 10 mL of sterile 0·1 M $MgSO_4$ and sonicated for 20 min to remove bacteria from the roots. Serial dilutions (10^2–10^4) of the suspension were plated onto BSV8A or KBA^{+++}, as appropriate, and incubated as described above to determine the number of cfu/g of root. There were six replicate plastic tubes for each experimental combination.

Effect of incubation temperature on bacterial growth and antagonistic activity against *F. oxysporum* f. sp. *ciceris*

Effect on growth and pyoverdine production by Pseudomonas fluorescens *isolates*

Bacterial cells in 100 μL suspension were transferred into 100-mL Erlenmeyer flasks with 40 mL of King's B broth (KB) at pH 7·0 (RGAF 19 and RG 26 isolates), or potato dextrose broth (PDB) (Difco Laboratories, Detroit, MI, USA) at pH 7·0 (RGAF 51 and RGAF 101 isolates) to obtain approximately 10^6 cells mL^{-1}. Cultures were incubated at each of nine temperatures, ranging from 15 to 37·5°C in 2·5°C increments on an orbital shaker at 125 rpm for 3 days. Bacterial cultures were centrifuged (10 000 ***g*** for 30 min) and supernatants adjusted to pH 7·0 were filtered twice through Millipore sterile filters (Millipore Corp., Bedford, MA, USA) of pore size 0·45 and 0·22 μm to eliminate any residual cells. Culture filtrates were plated onto PDA to check for viable bacteria before storage at −80°C until use. These filtered supernatants are referred to as crude filtrates to differentiate them from filtrates that were autoclaved (20 min at 121°C).

Culture pellets were resuspended in 10 mL sterile distilled water and the bacterial concentration was determined using standard curves as described above. Additionally, the concentration of the pyoverdine siderophore in supernatants from the *P. fluorescens* isolates was determined as previously described (Meyer & Abdallah, 1978; Landa *et al*., 2002). There were four replicate flasks for each experimental combination. The experiment was conducted in three separate assays, including three temperatures in each run plus 25°C as a common temperature treatment among them to allow for comparison among assays.

In vitro *radial growth inhibition of* Fusarium oxysporum *f. sp*. ciceris *by biocontrol agents*

Bacterial isolates were tested for their ability to inhibit fungal growth in dual cultures as previously described (Landa *et al*., 2001), using KBA and PDA at pH 7·0. Two bacteria (*P. fluorescens* RGAF 19 and *P. fluorescens* RG 26, or *B. megaterium* RGAF 51 and *P. macerans* RGAF 101) were spotted 1 cm from opposite edges of a plate and incubated at each of nine temperatures ranging from 12·5 to 35°C in 2·5°C increments for 48 h. An agar plug consisting of the fungal isolate grown on KBA or PDA at 25°C was then placed between the bacterial isolates in the centre of the plate containing the corresponding medium. The bacterial and fungal isolates were plated separately as controls. Cultures were incubated at each of the assayed temperatures for 6 days and the length of hyphal growth towards the bacteria (R_b) was measured and compared with that of a control plate (R_c). Inhibition of hyphal growth was recorded as percentage inhibition [RG-INH(%) = $100 \times (R_c - R_b)/R_c$]. There were five replicate plates for each experimental combination. The experiment was conducted in two separate assays, with 25 and 30°C as common temperature treatments between them to allow comparison between assays.

Effect of bacterial cell-free culture filtrates on in vitro *fungal growth*

Bacterial cell-free filtrates were obtained at each of seven temperatures ranging from 20 to 35°C as described above. Crude or autoclaved filtrates (50 μL) were added to wells in sterile flat-bottom microtitre plates containing 100 μL of a conidial suspension (10^3 conidia mL^{-1}) in modified KB medium (M-KB; 20 g of proteose peptone replaced by 5 g of casamino acids; Difco). Wells with 50 μL of sterile or double-sterilized PDB or KB broth and 100 μL of the conidial suspension in M-KB served as controls. Cultures were incubated at 25°C for 96 h, and absorbance of cultures at 630 nm was monitored at 3- to 5-h intervals with a microplate reader (ELx Bio-Tek Instruments, Inc., Winooski, VT, USA). Data of cumulative absorbance over time were plotted to obtain absorbance increase curves (AICs), and the area under the AICs (AUAIC) was used to indicate total fungal growth in the wells (Landa *et al*., 2002).

The same filtrates as above (50 μL) were added to wells containing 150 μL of a conidial suspension in sterile distilled water (10^5 conidia per well). The mixtures were incubated at 25°C for 12 h, then 10 μL of acid fuchsin in lactophenol was added to each well in the microplate and the percentage of germinated conidia [CG(%)] in 50 μL drops was

determined with a light microscope at ×400 magnification in five randomly chosen microscope fields (15 ± 6 conidia per field). A conidium was considered germinated when the germ tube length was greater than the conidial width.

For both fungal growth and conidial germination assays, there were three replicated wells for each experimental combination. A relative fungal growth inhibition index [FG-INH(%)] and a relative conidial germination inhibition index [CG-INH(%)] were calculated by the equation $RR = [(V_C - V_F)/V_C] \times 100$, where RR represents FG-INH(%) or CG-INH(%), V_C represents the average AUAIC or CG(%) values in the control treatment, and V_F represents the same values in the different treatments for the crude or sterilized bacterial filtrates.

Data analyses

All data analyses were conducted using the Statistical Analysis Software System (SAS Institute, Cary, NC, USA). Similarity between repeated experiments, tested by analysis of variance using experimental runs as blocks and Barlett's test of equal variances, allowed combining of data for analysis. In all experiments, a split-plot design was used in which temperatures were main plots, bacterial isolates were subplots, and trays, pots, Petri plates, flasks or wells in a microplate were replications. All experiments were repeated once except for those on antifungal activity of culture filtrates, which were repeated twice.

The effect of bacterial isolates on chickpea seedling emergence and growth and bacterial colonization of the plant rhizosphere were determined by multivariate and standard analysis of variance using the general linear model (GLM) procedure of SAS. Trend analysis, based on orthogonal polynomials for treatments with equal intervals for incubation temperature, was conducted if the incubation temperature had a significant ($P < 0{\cdot}05$) effect. In addition, orthogonal single-degree-of-freedom contrasts were computed to test the effects of bacterial treatments. Root colonization data were transformed to log(cfu/g of root) to satisfy assumptions of the statistical test used.

The effect of temperature on fungal growth and conidial germination inhibition by bacterial isolates was described by a modified beta function (Hau, 1988) fitted to the corresponding inhibition index using nonlinear regression analysis. The modified beta function can be written as:

$$\mathrm{INH}(\%) = \mathrm{INH}(\%)_{max} \times [(T - T_{min})/(T_{opt} - T_{min})]^{[B(T_{opt}-T_{min})/(T_{max}-T_{opt})]} \times [(T_{max} - T)/(T_{max} - T_{opt})^{B}]$$

In this function, T_{max} and T_{min} were fixed at 37 and 5°C, respectively, the known maximum and minimum temperatures for growth of *F. oxysporum* f. sp. *ciceris* isolate Foc-8012 (Duro-Almazán, 2000). The shape parameter B determines the temperature range near the optimum temperature (T_{opt}) at which INH(%) values are close to $\mathrm{INH}(\%)_{max}$ (maximum inhibition). Nonlinear regression analyses were conducted by the SAS least-squares procedure for nonlinear models (NLIN) as described before (Campbell & Madden, 1990; Landa *et al*., 2001). The standard errors of the parameter estimates in the regression analyses were used for comparison of the effects of culture media and the thermal stability of the bacterial cell-free filtrates on fungal inhibition (Campbell & Madden, 1990).

Results

Effect of incubation temperature on chickpea seedling emergence, growth promotion, and rhizosphere and stem colonization by rhizobacteria

Effect of temperature and bacterial treatments on chickpea emergence in sterilized and pasteurized soil

Emergence of chickpea seedlings was influenced by incubation temperature, bacterial treatment, heat treatment (sterilization or pasteurization) of the soil mixture, and their interactions (Table 1). In general, seedlings in the sterilized soil mixture emerged faster and in higher percentages than seeds sown in pasteurized soil, irrespective of incubation temperature (Table 1).

Seed and soil treatment with *B. megaterium* RGAF 51 or *P. macerans* RGAF 101 incubated at 20 or 30°C significantly ($P < 0{\cdot}05$) delayed and decreased seedling emergence from the sterilized soil mixture compared with that in nontreated controls. At the 30°C incubation temperature, all bacterial treatments significantly ($P < 0{\cdot}05$) delayed the time to chickpea seedling emergence (t_e) from the sterilized soil mixture compared with that of the control treatment (Table 1). In the pasteurized soil mixture, there were no significant differences ($P = 0{\cdot}05$) among the bacterial and control treatments, except for *P. fluorescens* RG 26. Seed and soil treatments with *P. fluorescens* RG 26 significantly ($P < 0{\cdot}05$) shortened $t_{50\%}$ and increased percentage seedling emergence (both FE and SAUEIC) at 20°C. Multivariate analysis of variance indicated that the overall effect of all bacterial treatments on seedling emergence (as determined by t_e, $t_{50\%}$, FE and SAUEIC) at 20°C, and that of *P. macerans* RGAF 101 at 25 and 30°C and *P. fluorescens* RGAF 19 at 25°C, was significantly ($P < 0{\cdot}05$) different when seeds were sown in the sterilized soil mixture rather than pasteurized soil (data not shown).

Seed and soil treatment with *P. fluorescens* RGAF 19 resulted in the time to reach 50% emergence ($t_{50\%}$) being significantly ($P < 0{\cdot}05$) shorter for seeds sown in sterilized soil mixture compared with seeds sown in pasteurized soil at 20 and 25°C (Table 1). In contrast, treatment with *P. fluorescens* RG 26 at 20 or 25°C and *P. macerans* RGAF 101 at 30°C significantly ($P < 0{\cdot}05$) increased $t_{50\%}$ in the sterilized soil mixture compared with that in the pasteurized mixture (Table 1). Similarly, seedling emergence, determined by final percentage emergence (FE) and the standardized area under the emergence increase curve (SAUEIC), was larger in the sterilized soil mixture than in the pasteurized one. However, the net effect of soil heat treatment was influenced by both bacterial treatment and incubation temperature. FE was significantly higher ($P < 0{\cdot}05$) in the sterilized soil mixture than in the pasteurized one for all bacterial treatments incubated at 20, 25 or

Table 1 Effect of incubation temperature and bacterial treatments on emergence of chickpea seedlings from sterilized and pasteurized soil

Temperature (°C)	Bacterial treatment[a]	Seedling emergence variables[b]			
		t_e (h)	$t_{50\%}$ (h)	*FE* (%)	SAUEIC
Sterilized soil					
20	Control	65·1	82·5	100	90·7
	Pf RGAF 19	59·4	83·7	100	86·1
	Pf RG 26	70·5	91·7*	95·8*	84·7
	Bm RGAF 51	74·6	99·1*	95·8*	78·0*
	Pm RGAF 101	72·5	94·0*	97·9*	86·0
25	Control	63·5	81·7	100	90·0
	Pf RGAF 19	56·9	79·2	100	91·8
	Pf RG 26	64·4	83·3	95·8*	88·9
	Bm RGAF 51	69·3	87·1	87·5*	81·2
	Pm RGAF 101	60·3	77·0	97·9*	92·0
30	Control	28·1	74·6	97·9	84·1
	Pf RGAF 19	44·6*	71·8	89·6	85·5
	Pf RG 26	50·4*	72·8	97·9	92·3
	Bm RGAF 51	53·6*	85·2*	75·0*	66·1*
	Pm RGAF 101	60·3*	79·7	100*	89·1
Pasteurized soil					
20	Control	57·8	90·7	77·1	67·1
	Pf RGAF 19	61·9	94·9	75·0	64·7
	Pf RG 26	61·9	81·2*	97·9*	84·5*
	Bm RGAF 51	75·0	103·6*	54·2	48·1
	Pm RGAF 101	69·5	89·4	66·7	61·6
25	Control	52·0	82·1	83·3	74·0
	Pf RGAF 19	61·0	80·6	66·7	64·5
	Pf RG 26	57·0	75·4	93·8	87·6
	Bm RGAF 51	65·1	88·8	60·4	54·4
	Pm RGAF 101	54·5	82·7	66·7	62·2
30	Control	44·6	73·4	75·0	71·5
	Pf RGAF 19	48·8	68·6	72·9	76·6
	Pf RG 26	48·8	67·4	85·4	85·9
	Bm RGAF 51	52·9	81·0	66·7	62·3
	Pm RGAF 101	48·8	72·9	75·0	72·6

[a]*Pseudomonas fluorescens* RGAF 19, *P. fluorescens* RG 26, *Bacillus megaterium* RGAF 51 and *Paenibacillus macerans* RGAF 101 were applied as seed and soil treatments.
[b]t_e, time in hours to reach seedling emergence > 0%; $t_{50\%}$, time in hours to reach emergence = 50%; FE, final percentage emergence; SAUEIC, standardized area under the emergence increase curve. Underlined means in a treatment in the sterilized soil are significantly ($P < 0{\cdot}05$) different from the same treatment in pasteurized soil; means followed by an asterisk (*) are significantly different ($P < 0{\cdot}05$) from those of the control treatment according to linear single-degree-of-freedom contrasts of analysis of variance.

30°C, except for *P. fluorescens* RG 26 at 20 and 25°C and *B. megaterium* RGAF 51 at 25 and 30°C.

SAUEIC was significantly higher ($P < 0{\cdot}05$) in the sterilized soil mixture than in the pasteurized one for all bacterial treatments at 20 and 25°C, except for *P. fluorescens* RG 26 at those two temperatures and *P. fluorescens* RGAF 19 at 20°C. No significant ($P = 0{\cdot}05$) differences between soil heat treatments were observed at 30°C (Table 1).

Incubation temperature had a major effect both on t_e and $t_{50\%}$. Thus, chickpea seedling emergence at 30°C occurred 21 h earlier and reached 50% 13·5 h sooner than at 20°C in the sterilized soil mixture, and 17 h earlier and 20 h sooner in the pasteurized one (Table 1). On average, t_e and $t_{50\%}$ decreased linearly ($P < 0{\cdot}05$) with increase in temperature from 20 to 30°C. This linear trend accounted for 61·3–99·6% of variation in t_e and $t_{50\%}$ in the sterilized soil mixture, and 80·0–99·9% of that in the pasteurized one (data not shown).

Table 2 Effect of incubation temperature and treatment with bacterial isolates on growth of chickpea

Temperature (°C)	Bacterial treatment[a]	Chickpea growth variables[b]		
		Stem length (cm)	Shoot dry weight (g)	Root dry weight (g)
20	Control	31·06	2·93	1·74
	Pf RGAF 19	30·63	3·81*	1·94
	Pf RG 26	32·23	3·83*	1·96
	Bm RGAF 51	33·09	3·58	1·60
	Pm RGAF 101	31·66	4·04*	1·46
25	Control	27·66	2·84	1·29
	Pf RGAF 19	29·40	2·99	1·96
	Pf RG 26	32·49	3·48	2·39*
	Bm RGAF 51	29·48	3·03	1·46
	Pm RGAF 101	29·27	3·08	1·70
30	Control	27·89	2·60	1·14
	Pf RGAF 19	29·36	2·74	2·10*
	Pf RG 26	28·71	2·76	2·47*
	Bm RGAF 51	27·28	2·69	1·81
	Pm RGAF 101	25·41	2·35	1·56

[a]*Pseudomonas fluorescens* RGAF 19, *P. fluorescens* RG 26, *Bacillus megaterium* RGAF 51 and *Paenibacillus macerans* RGAF 101 were applied as seed and soil treatments.
[b]Chickpea growth was assessed at the end of the experiment, 45 days after sowing. Means followed by an asterisk (*) are significantly different ($P < 0{\cdot}05$) from those of the controls according to linear single-degree-of-freedom contrasts of analysis of variance.

Effect of temperature on chickpea growth

Growth of chickpea cv. PV 61 was influenced mainly by incubation temperature and, to a lesser extent, by bacterial treatment as well as by their interactions. Results were similar for fresh and dry stem and root weights; therefore, only data for dry weights are presented (Table 2). Multivariate analysis of variance indicated that combined seed and soil treatment with *P. macerans* RGAF 101 at 20°C, *P. fluorescens* RG 26 at 25 or 30°C, or *P. fluorescens* RGAF 19 at 30°C significantly ($P < 0{\cdot}05$) promoted chickpea growth (as determined by stem length and shoot and root dry weights) compared with nontreated controls (data not shown). That effect was mainly caused by the increase in shoot dry weight induced by *P. macerans* RGAF 101. Increase in root dry weight resulted from treatments with *P. fluorescens* RGAF 19 and RG 26 (Table 2). In general, the bacterial isolates in the study either increased or had no deleterious effect on chickpea growth. However, only treatments with *P. fluorescens* isolates significantly ($P < 0{\cdot}05$) increased plant growth compared with the control at all incubation temperatures, as well as with *P. macerans*

RGAF 101 and *B. megaterium* RGAF 51 at 30°C. On average, treatments with both *P. fluorescens* strains increased stem length, shoot dry weight and root dry weight by 5·7, 16·7 and 60·4%, respectively, compared with controls (Table 2).

Incubation temperature did not have a significant ($P = 0{\cdot}05$) effect on stem length of plants grown in the *P. fluorescens* and control treatments. However, treatments with *P. macerans* RGAF 101 and *B. megaterium* RGAF 51 resulted in a negative linear trend ($P < 0{\cdot}05$) of stem length with temperature increase from 20 to 30°C that accounted at least for 98% of the variation in chickpea stem length with temperature (data not shown). Shoot dry weight in the control treatment was not affected ($P = 0{\cdot}05$) by incubation temperature. In contrast, all bacterial treatments resulted in a negative linear trend ($P < 0{\cdot}05$) of shoot dry weight with temperature increase, which accounted for 91·3–99·3% of the variation in chickpea dry stem weight with temperature (data not shown). There were no significant differences ($P = 0{\cdot}05$) in root dry weight with respect to incubation temperature (data not shown).

Effect of temperature on colonization of stems and rhizosphere by introduced rhizobacteria

The effect of temperature on bacterial rhizosphere colonization varied with the bacterial isolate and application method used. Rhizosphere colonization by all bacterial isolates was significantly higher ($P < 0{\cdot}05$) in combined seed and soil (SE + SO) treatments than in seed-only treatments (SE). For SE + SO treatments, mean rhizosphere colonizations by *P. fluorescens* RGAF 19, *P. fluorescens* RG 26, *B. megaterium* RGAF 51 and *P. macerans* RGAF 101 were log 7·56, 7·75, 7·42 and 6·75 cfu/g of root, respectively, compared with log 7·23, 7·42, 7·04 and 6·37 cfu/g of root in the respective SE treatments. For most treatments, there was a positive linear trend ($P < 0{\cdot}05$) in bacterial rhizosphere colonization with increase in temperature from 20 to 30°C, accounting for 60–99% of variation (data not shown). This general trend did not occur for the SE treatments with *B. megaterium* RGAF 51 and *P. macerans* RGAF 101, or the SE + SO treatment with *P. fluorescens* RGAF 19. For those treatments, there was a significant quadratic trend ($P < 0{\cdot}05$) with increase in temperature, with the greatest extent of rhizosphere colonization occurring at 25°C.

All bacterial isolates colonized the stem tissue of the chickpeas endophytically at all the incubation temperatures studied, but the extent of colonization, estimated as the percentage of stem pieces colonized, varied according to temperature, bacterial isolate and the stem internode sampled. Percentage isolation of bacteria from internal stem tissues was highest (> 68%) in the lowest internode sampled and at 25°C. Nevertheless, at this temperature all bacteria were recovered from the uppermost chickpea stem internodes in more than 3% of isolations. The extent of endophytic colonization decreased with acropetal progression of sampled stem tissues. No bacteria were isolated from the uppermost stem internode at 20 or 30°C, except for a small percentage (3%) in plants treated with *P. fluorescens* RGAF 19 or *B. megaterium* RGAF 51 at 30°C.

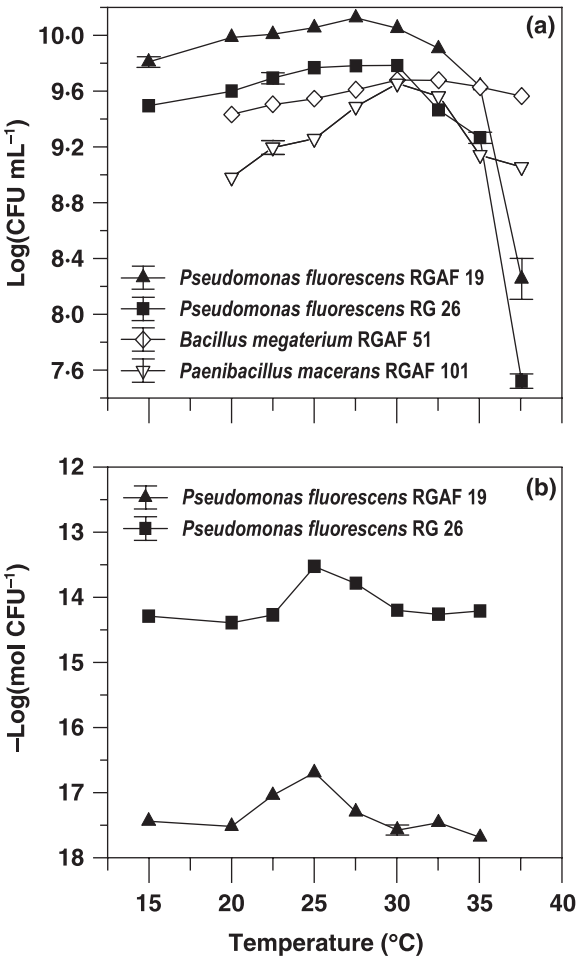

Figure 1 (a) Growth of bacterial isolates in liquid medium at different temperatures. (b) Pyoverdine production by *Pseudomonas fluorescens* isolates at different temperatures. Vertical bars represent standard errors.

Effect of incubation temperature on bacterial growth and antagonistic activity against *F. oxysporum* f. sp. *ciceris* race 5

Effect on bacterial growth and pyoverdine production by Pseudomonas fluorescens *isolates*

The optimum temperature for bacterial growth differed among isolates. *Bacillus megaterium* RGAF 51 and *P. macerans* RGAF 101 did not grow in PDB at 15°C but grew best at 27·5–35°C. The optimum temperature for growth of *P. fluorescens* RGAF 19 and *P. fluorescens* RG 26 in KB broth was 25–32·5°C (Fig. 1a). *Pseudomonas fluorescens* isolates grew in KB broth at all incubation temperatures, but to a lesser extent at 37·5°C, the highest temperature tested (Fig. 1a). *Pseudomonas fluorescens* isolates produced the pyoverdine siderophore in KB broth at all incubation temperatures except 37·5°C. The amount of pyoverdine in the medium increased with

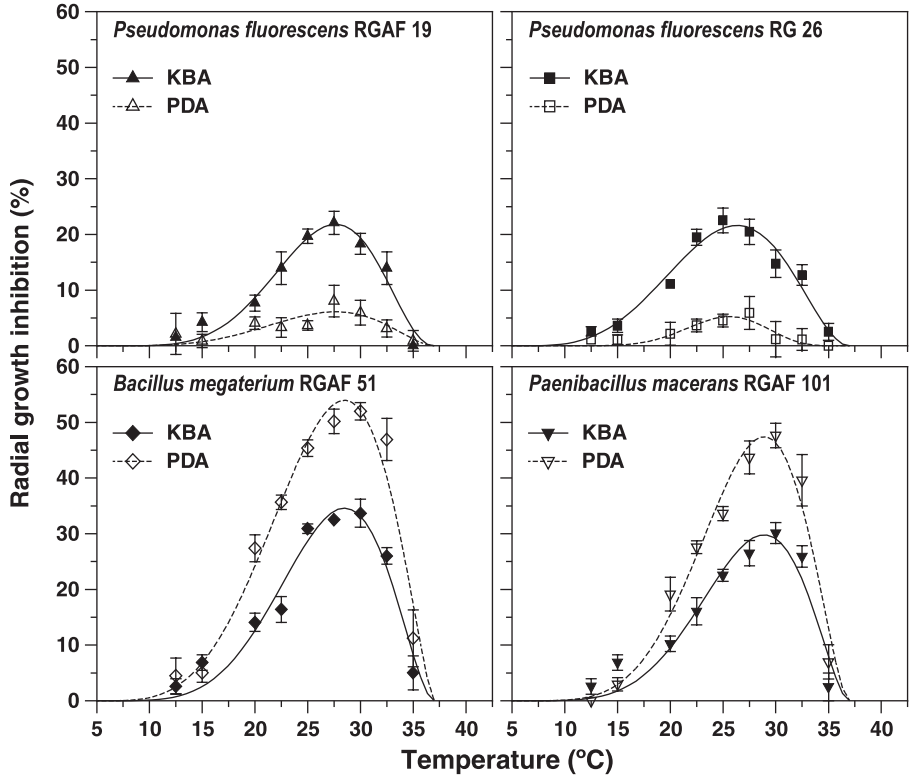

Figure 2 Effect of incubation temperature on radial growth inhibition (%) of *Fusarium oxysporum* f. sp. *ciceris* race 5 radial growth by four bacterial isolates on King's B medium agar (KBA) and potato dextrose agar (PDA). Curves show the modified beta function fitted to data. Vertical bars represent standard errors.

incubation temperature as it approached the optimum for bacterial growth (Fig. 1). The highest total pyoverdine production [log(mol L^{-1})] occurred at 27·5°C for *P. fluorescens* RGAF 19 and at 25°C for *P. fluorescens* RG 26 (data not shown). However, both these isolates produced the highest relative amount of pyoverdine [log(mol $cell^{-1}$)] at 25°C (Fig. 1b).

Effect on in vitro Fusarium oxysporum *f. sp.* ciceris *radial growth inhibition and antifungal activity of bacterial cell-free culture filtrates*

Inhibition of radial growth of *F. oxysporum* f. sp. *ciceris* race 5 was influenced by incubation temperature, bacterial isolate and culture medium (KBA, PDA) where dual cultures were established. Inhibition of fungal growth by bacterial isolates was significantly ($P < 0{\cdot}05$) influenced by temperature, irrespective of culture media. In all cases, variation in fungal growth inhibition with temperature was adequately described by the modified beta function (coefficient of determination $R^2 > 0{\cdot}79$) (Fig. 2). In general, radial growth of *F. oxysporum* f. sp. *ciceris* race 5 was greatly reduced by bacteria at temperatures ranging from 22·5 to 32·5°C (Fig. 2). However, for both KBA and PDA media, the temperature resulting in the greatest inhibition of fungal radial growth (T_{opt}) differed among bacterial isolates, being higher for *B. megaterium* RGAF 51 and *P. macerans* RGAF 101 than for the two *P. fluorescens* isolates (Fig. 2). The estimated mean T_{opt} was 25·9°C for *P. fluorescens* RG 26, 27·6°C for *P. fluorescens* RGAF 19, 28·5°C for *B. megaterium* RGAF 51 and 28·9°C for *P. macerans* RGAF 101, irrespective of culture media. The temperature range for maximum radial growth inhibition, as indicated by *B* parameter estimates, was broader for *P. fluorescens* isolates ($B = 1{\cdot}87–5{\cdot}77$) than for *B. megaterium* RGAF 51 and *P. macerans* RGAF 101 ($B = 1{\cdot}47–1{\cdot}85$). However, the two isolates of *P. fluorescens* inhibited fungal radial growth to a lesser extent than *B. megaterium* RGAF 51 and *P. macerans* RGAF 101, irrespective of culture media (Fig. 2). With regard to the culture media, inhibition of radial growth of *F. oxysporum* f. sp. *ciceris* race 5 by *P. fluorescens* isolates was almost nil on PDA [RG-INH$(\%)_{max} < 6{\cdot}2\%$] and significantly ($P < 0{\cdot}05$) higher on KBA [RG-INH$(\%)_{max} > 21{\cdot}4\%$]. Conversely, fungal radial growth inhibition by *B. megaterium* RGAF 51 and *P. macerans* RGAF 101 was significantly ($P < 0{\cdot}05$) higher on PDA [RG-INH$(\%)_{max} > 47{\cdot}4\%$] than on KBA [RG-INH$(\%)_{max} > 29{\cdot}8\%$] (Fig. 2).

Inhibition of *F. oxysporum* f. sp. *ciceris* race 5 conidial germination and mycelial growth by the bacterial cell-free culture filtrates was influenced by incubation temperature and the nature of the culture filtrate. The inhibitory activities of all bacterial culture filtrates decreased significantly ($P < 0{\cdot}05$) after autoclaving (121°C for 20 min), indicating that filtrates were somewhat thermolabile (Fig. 3). For

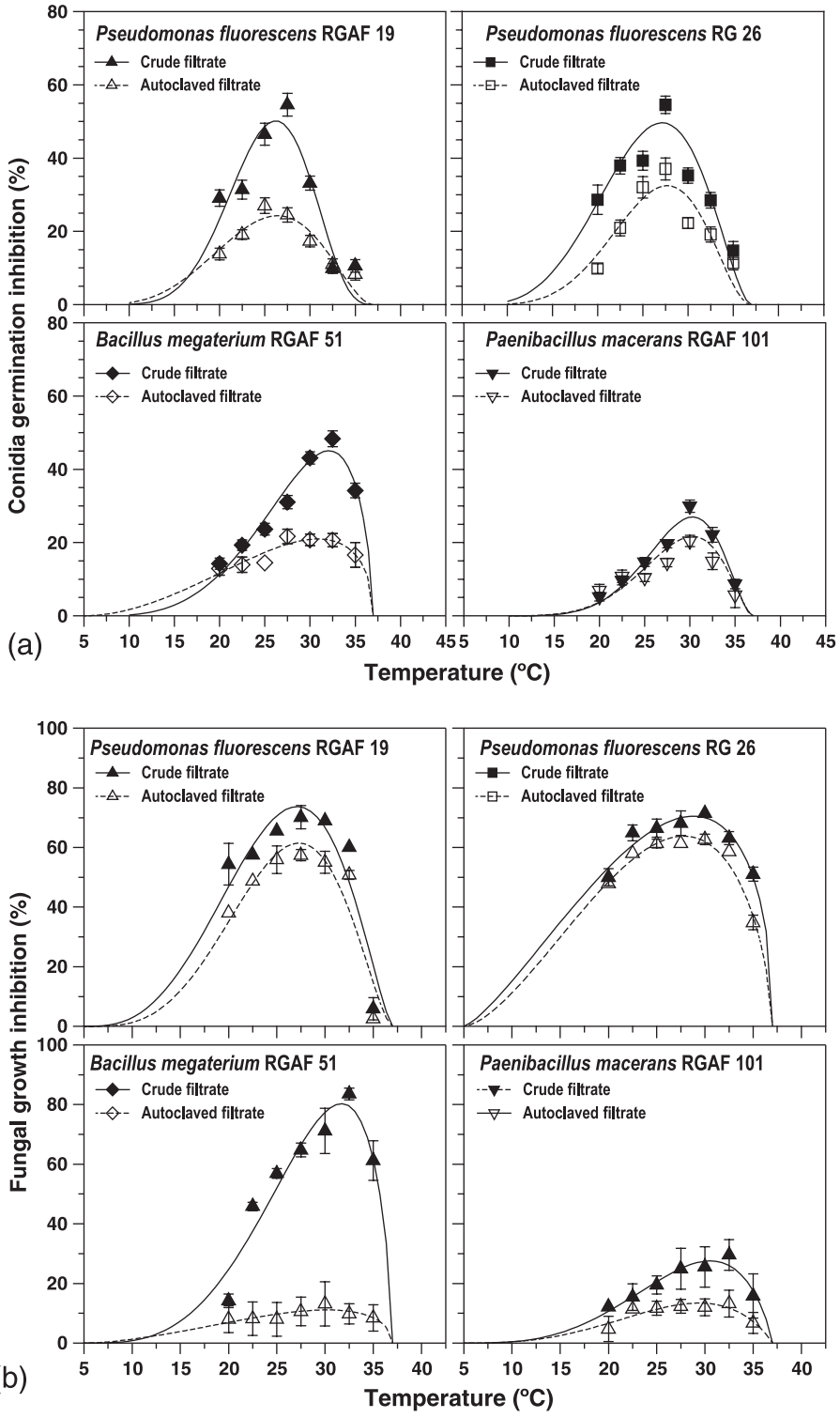

Figure 3 Effect of cell-free crude or autoclaved (120°C, 20 min) culture filtrates of four bacterial isolates obtained at different temperatures on inhibition of conidial germination (a) and fungal growth of *Fusarium oxysporum* f. sp. *ciceris* race 5 (b). Curves show the modified beta function fitted to data. Vertical bars represent standard errors.

all experimental combinations, the effect of temperature on the inhibitory activity exhibited by the bacterial culture filtrates on conidial germination and mycelial growth was adequately described by the modified beta function ($R^2 > 0{\cdot}78$) (Fig. 3). For each bacterial isolate, maximum inhibition of conidial germination (CG-INH$(\%)_{max}$) and mycelial growth [FG-INH$(\%)_{max}$] occurred at a similar temperature (T_{opt}). However, T_{opt} differed among bacterial isolates, being higher for *B. megaterium* RGAF 51 and *P. macerans* RGAF 101 (29·4–32·2°C) than for the two *P. fluorescens* isolates (26·2–28·8°C) (data not shown). Overall, the inhibition of conidial germination and mycelial growth of *F. oxysporum* f. sp. *ciceris* race 5 at T_{opt} was significantly ($P < 0{\cdot}05$) higher for the bacterial crude filtrates then the autoclaved filtrates (Fig. 3). The maximum difference between inhibitory activities of crude and autoclaved filtrates occurred with *B. megaterium* RGAF 51, for which CG-INH$(\%)_{max}$ and FG-INH$(\%)_{max}$ values were 2·1 and 7·2 times higher, respectively, with the crude filtrate than with the autoclaved filtrate (data not shown). The lowest inhibition of conidial germination and mycelial growth occurred with either crude or autoclaved filtrates of *P. macerans* RGAF 101 (Fig. 3).

Discussion

In previous research (Landa *et al*., 2001) on the fusarium wilt/chickpea pathosystem, seed and soil treatment with the rhizobacteria used in the present study (especially the two *P. fluorescens* isolates) suppressed fusarium wilt by delaying the development of disease symptoms and reducing the rate of disease increase at 20 and 30°C, but not at 25°C (Landa *et al*., 2001). In the absence of rhizobacteria, higher numbers of *F. oxysporum* f. sp. *ciceris* infections were necessary at 20 and 30°C than at 25°C for disease to develop early, fast and severely. The present study mainly investigated the effects of those temperatures on activities of rhizobacteria in relation to efficiency of suppression of fusarium wilt (Burpee, 1990; Beauchamp *et al*., 1991). The temperatures studied (20, 25 and 30°C) were chosen because: (i) they comprise the optimum temperatures for chickpea germination, seedling emergence and growth (Covell *et al*., 1986; Özdemir, 1996; Singh & Virmani, 1996); and (ii) they are within the range of mean soil temperatures that occur during the crop cycle (mid-March to late June) of spring chickpea sowings in the Mediterranean region and favour fusarium wilt incidence and severity (Navas-Cortés *et al*., 1998).

The four rhizobacteria in the present study behaved as endophytes since they colonized internal chickpea tissues without harming the plant (Hallmann, 2001), and such endophytic colonization can result both from seedborne and soilborne inocula (Kim *et al*., 1997; Hallmann, 2001; Wulff *et al*., 2002). The colonization of internal chickpea tissues by the rhizobacteria in the present study provided them with a relatively uniform and protected environment, possibly gaining them some advantages as biocontrol agents of xylem-colonizing *F. oxysporum* f. sp. *ciceris* by avoiding competition with other microorganisms for nutrients. However, the specific bacterial traits that confer endophytic ability in the chickpea stem, as well as the relationships that may exist between that ability and their potential for suppression of fusarium wilt of chickpea, have yet to be determined.

Production of antifungal compounds and siderophores is a primary mechanism of disease suppression by *Bacillus* spp. and fluorescent *Pseudomonas* spp. (Edwards *et al*., 1994; Wulff *et al*., 2002). However, biosynthesis of these metabolites is modulated by a number of biotic and abiotic factors (Duffy & Défago, 1999; Raaijmakers *et al*., 2002). The results of the present study indicate that temperature significantly influences the ability of bacteria to produce inhibitory metabolites in culture media. The interacting microorganisms in the study differed in their temperature ranges for optimum growth. However, those temperature ranges allowed rather similar optimum temperatures for both bacterial growth and inhibition of pathogen radial growth. Similar to the interactions found in dual cultures, the production of inhibitory metabolites by bacteria in liquid cultures was higher at the optimum growth temperature, suggesting similar optimum temperatures for growth and production of antifungal metabolites. For the two *P. fluorescens* isolates in this study, production of the pyoverdine siderophore was higher at the same optimum temperature for growth and repressed at 37·5°C, when bacterial growth decreased. The results of this study agree with those of other authors in that production of siderophores by *Pseudomonas* spp. was greatest at 25–27°C (Barton *et al*., 1996) and was repressed at 37°C (Marugg *et al*., 1985).

The antifungal effect of the bacterial cell-free culture filtrates suggests the presence of more than a single compound, since the filtrates showed a residual inhibitory activity after autoclaving, except for those of *B. megaterium* RGAF 51, which must have contained heat-sensitive metabolites. Heat-stable metabolites have been isolated from culture filtrates of other rhizobacteria (Pusey & Wilson, 1984; Edwards *et al*., 1994). Both *P. fluorescens* isolates showed low inhibitory activity of mycelial growth of *F. oxysporum* f. sp. *ciceris*. However, cell-free KB broth culture filtrates of these isolates inhibited conidial germination of the pathogen to a similar extent as those of *B. megaterium* RGAF 51 and more than those of *P. macerans* RGAF 101. This suggests that activity of *P. fluorescens* cell-free filtrates against the pathogen may consist primarily of inhibition of conidial germination and/or early germ tube elongation. Inhibition of conidial germination as a main antifungal activity was reported for siderophores produced by fluorescent *Pseudomonas* spp. as a result of siderophore-mediated competition for iron, and also in the case of antibiotics produced by *Bacillus* sp. (Loper & Schroth, 1986; Lemanceau *et al*., 1993; Edwards *et al*., 1994). The ability of the four rhizobacteria in the present study to produce extracellular metabolites inhibitory of *F. oxysporum* f. sp. *ciceris* race 5 growth and conidial germination suggests that production of antifungal metabolites in the rhizosphere might be associated with previously reported disease suppression by those

rhizobacteria (Landa *et al*., 2001). Nevertheless, *in vitro* antagonism by microorganisms does not necessarily guarantee that they can be effective in disease suppression (Weller & Thomashow, 1994).

In many cases, inconsistency in disease suppression by introduced rhizobacteria has been attributed to limited colonization of the plant rhizosphere (Weller, 1988; Mahaffee & Backman, 1993). The present study found that populations of the introduced bacteria in the chickpea rhizosphere increased linearly with temperature (from 20 to 30°C) by 10 days after sowing. Published results of temperature effect on rhizosphere colonization by bacterial inoculants are sparse and conflicting. Some studies indicated better colonization by introduced bacteria at high temperature (Davies & Whitbread, 1989), but the opposite effect occurred in most cases, i.e. rhizosphere colonization by *Pseudomonas* strains was best at 12–20°C (Loper *et al*., 1985; Weller, 1988; Bowers & Parke, 1993; Weller & Thomashow, 1994) despite the fact that *in vitro* bacterial growth was optimal at 25–30°C. Better rhizosphere colonization by the introduced bacteria at low temperature probably indicates that competition from indigenous soil microbial activity declines with temperature (Beauchamp *et al*., 1991; Weller & Thomashow, 1994).

Root-colonizing *Bacillus* and *Pseudomonas* spp. can promote plant growth, but failure to do so may occur and has been attributed to crop- or cultivar-specific activity, differences in root colonization by the introduced rhizobacteria, or inappropriate environmental conditions (Davies & Whitbread, 1989; Turner & Backman, 1991; Mahafee & Backman, 1993; Weller & Thomashow, 1994; Smith & Goodman, 1999). The results of the present study showed that at optimum temperatures for chickpea emergence and growth (20–30°C) all the bacterial isolates increased, or had no deleterious effect on, chickpea growth. However, only *P. fluorescens* isolates significantly increased root dry weight, especially at 25 and 30°C. The nonsignificant plant growth promotion occurring in some cases in this study might relate to the use of a pasteurized mixture lacking deleterious microorganisms, since competition with deleterious microorganisms has been suggested as having a major role in plant growth promotion by rhizobacteria (Davies & Whitbread, 1989). Interestingly, fusarium wilt of chickpea was suppressed by these rhizobacteria at 20 or 30°C, but not at 25°C, the temperature at which disease developed most severely (Landa *et al*., 2001). It appeared that at 25°C the disease potential was just too high to be countered by the biocontrol agent (Landa *et al*., 2001). Of particular interest is that the greatest levels of disease suppression were obtained at 30°C (Landa *et al*., 2001), the temperature at which rhizosphere colonization and production of inhibitory metabolites by the rhizobacteria were close to the highest values found and, in the present study, chickpea root dry weight and rate of seedling emergence increased.

In conclusion, it has been shown in this study that activities of rhizobacteria that may be of importance in the biocontrol of fusarium wilt of chickpea are significantly affected by incubation temperature, as is the actual degree of disease suppression achieved (Landa *et al*., 2001). Such activities include those related to direct antagonism of the rhizobacteria against the pathogen, such as production of antifungal metabolites, or activities that affect the interaction between the host plant and the rhizobacteria and thus might have an indirect effect on the pathogen, such as rhizosphere and stem colonization, and growth promotion. The results emphasize the need for better understanding of the effect of environmental factors on the biological activities of rhizobacteria introduced for disease suppression. Identification of environmental factors that influence the effectiveness of these rhizobacteria in terms of disease control would provide a basis for improved integration of biocontrol treatments with other control practices that have shown effectiveness in the management of fusarium wilt of chickpea (Navas-Cortés *et al*., 1998), including choice of sowing date and use of partially resistant cultivars.

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References

Adams PB, Ayers WA, 1980. Factors affecting parasitic activity of *Sporidesmium sclerotivorum* on sclerotia of *Sclerotinia minor* in soil. *Phytopathology* **70**, 366–8.

Barton HA, Johnson Z, Cox CD, Vasil AI, Vasil ML, 1996. Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression exotoxin A and siderophores in aerobic and microaerobic environments. *Molecular Microbiology* **21**, 1001–17.

Beauchamp CJ, Kloepper JW, Chalifour FP, Antoun H, 1991. Effects of temperature on growth promotion and root colonization of potato by bioluminescent rhizobacteria mutants. *Plant Growth Promoting Rhizobacteria: Progress and Prospects. Bulletin OILB* **14**, 332–5.

Bowers JH, Parke JL, 1993. Colonization of pea (*Pisum sativum* L.) taproots by *Pseudomonas fluorescens*: effect of soil temperature and bacterial motility. *Soil Biology and Biochemistry* **25**, 1693–701.

Burpee LL, 1990. The influence of abiotic factors on biological control of soilborne plant pathogenic fungi. *Canadian Journal of Plant Pathology* **12**, 308–17.

Campbell CL, Madden LV, 1990. *Introduction to Plant Disease Epidemiology*. New York: John Wiley.

Covell S, Ellis RH, Roberts EH, Summerfield RJ, 1986. The influence of temperature on seed germination rate in grain legumes. I. A comparison of chickpea, lentil, soybean and cowpea at constant temperatures. *Journal of Experimental Botany* **37**, 705–15.

Davies KG, Whitbread R, 1989. Factors affecting the colonization of a root system by fluorescent pseudomonads: the effects of water, temperature, and soil microflora. *Plant and Soil* **116**, 247–56.

Duffy BK, Défago G, 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Applied and Environmental Microbiology* **65**, 2429–38.

Duro-Almazán MD, 2000. *Influencia de la Temperatura y el pH en el Desarrollo in vitro de Razas de* Fusarium oxysporum *f. sp.* ciceris. MSc Thesis. Córdoba, Spain: ETSIAM, Universidad de Córdoba.

Edwards SG, McKay T, Seddon B, 1994. Interaction of *Bacillus* species with phytopathogenic fungi. Methods of analysis and manipulation for biocontrol purposes. In: Blakeman JP, Williamson B, eds. *Ecology of Plant Pathogens*. Wallingford, UK: CAB International, 101–18.

Hallmann J, 2001. Plant interactions with endophytic bacteria. In: Jeger MJ, Spence NJ, eds. *Biotic Interactions in Plant–Pathogen Associations*. Wallingford, UK: CABI Publishing, 87–119.

Hau B, 1988. *Ein Erweitertes Analytisches Modell für Epidemien Von Pflanzenkrankheiten*. Giessen, Germany: Justus-Liebig-Universität, Habilitation-sschrift.

Henis Y, Papavizas GC, 1983. Factors affecting germinability and susceptibility to attack of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum* in field soil. *Phytopathology* **73**, 1469–74.

Kim D-S, Weller DM, Cook RJ, 1997. Population dynamics of *Bacillus* sp. L324-92R_{12} and *Pseudomonas fluorescens* 2-79RN_{10} in the rhizosphere of wheat. *Phytopathology* **87**, 559–64.

King EO, Ward MK, Raney DE, 1954. Two simple media for the demonstration of pyocyanine and fluorescein. *Journal of Laboratory and Clinical Medicine* **44**, 301–7.

Landa BB, Cachinero-Díaz JM, Lemanceau P, Jiménez-Díaz RM, Alabouvette C, 2002. Effect of fusaric acid and phytoanticipins on growth of rhizobacteria and *Fusarium oxysporum*. *Canadian Journal of Microbiology* **48**, 971–85.

Landa BB, Hervás A, Bettiol W, Jiménez-Díaz RM, 1997. Antagonistic activity of bacteria from chickpea rhizosphere against *Fusarium oxysporum* f. sp. *ciceris*. *Phytoparasitica* **25**, 305–38.

Landa BB, Navas-Cortés JA, Hervás A, Jiménez-Díaz RM, 2001. Influence of temperature and inoculum density of *Fusarium oxysporum* f. sp. *ciceris* on suppression of Fusarium wilt of chickpea by rhizosphere bacteria. *Phytopathology* **91**, 807–16.

Lemanceau P, Bakker PAHM, De Koger WJ, Alabouvette C, Schippers B, 1993. Antagonistic effect of nonpathogenic *Fusarium oxysporum* Fo47 and pseudobactin 358 upon pathogenic *Fusarium oxysporum* f. sp. *dianthi*. *Applied and Environmental Microbiology* **59**, 74–82.

Lewis JA, Papavizas C, 1985. Effect of mycelial preparations of *Trichoderma* and *Gliocladium* on populations of *Rhizoctonia solani* and the incidence of damping-off. *Phytopathology* **75**, 812–7.

Loper JE, Haack C, Schroth MN, 1985. Population dynamics of soil pseudomonads in the rhizosphere of potato (*Solanum tuberosum*, L.). *Applied and Environmental Microbiology* **49**, 416–22.

Loper JE, Schroth MN, 1986. Importance of siderophores in microbial interactions in the rhizosphere. In: Swinburne TR, ed. *Iron, Siderophores and Plant Diseases*. New York: Plenum Press, 85–9.

Mahaffee WF, Backman PA, 1993. Effects of seed factors on spermosphere and rhizosphere colonization of cotton by *Bacillus subtilis* GB03. *Phytopathology* **83**, 1120–5.

Marugg JD, van Spanje M, Hoekstra WPM, Schippers B, Weisbeek PJ, 1985. Isolation and analysis of genes involved in siderophore biosynthesis in plant-growth-stimulating *Pseudomonas putida* WCS358. *Journal of Bacteriology* **164**, 563–70.

Meyer JM, Abdallah MA, 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *Journal of General Microbiology* **107**, 319–28.

Munnecke DE, Wilbur W, Darley F, 1976. Effect of heating or drying on *Armillaria mellea* or *Trichoderma viride* and the relation to survival of *A. mellea* in soil. *Phytopathology* **66**, 1363–8.

Navas-Cortés JA, Hau B, Jiménez-Díaz RM, 1998. Effect of sowing date, host cultivar, and race of *Fusarium oxysporum* f. sp. *ciceris* on development of Fusarium wilt of chickpea. *Phytopathology* **88**, 1338–46.

Olsen CM, Baker KF, 1968. Selective heat treatment of soil, and its effect on the inhibition of *Rhizoctonia solani* by *Bacillus subtilis*. *Phytopathology* **58**, 78–87.

Ownley BH, Duffy BK, Weller DM, 2003. Identification and manipulation of soil properties to improve the biological control performance of phenazine-producing *Pseudomonas fluorescens*. *Applied and Environmental Microbiology* **69**, 3333–43.

Özdemir S, 1996. Effect of temperature on germination of chickpea. *International Chickpea and Pigeonpea Newsletter* **3**, 27–9.

Paulitz TC, Baker R, 1987. Biological control of Pythium damping-off of cucumbers with *Pythium nunn*: influence of soil environment and organic amendments. *Phytopathology* **77**, 341–6.

Pusey PL, Wilson CL, 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. *Plant Disease* **68**, 753–6.

Raaijmakers JM, Vlami M, de Souza JT, 2002. Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek* **81**, 537–47.

Simon A, Ridge EH, 1974. The used of ampicillin in a simplified selective medium for the isolation of fluorescent pseudomonads. *Journal of Applied Bacteriology* **37**, 459–60.

Singh P, Virmani SM, 1996. Modelling growth and yield of chickpea (*Cicer arietinum* L.). *Field Crop Research* **46**, 41–9.

Smith KP, Goodman RM, 1999. Host variation for interactions with beneficial plant-associated microbes. *Annual Review of Phytopathology* **37**, 473–91.

Turner JT, Backman PA, 1991. Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis*. *Plant Disease* **75**, 347–53.

Weller DM, 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology* 26, 379–407.

Weller DM, Thomashow LS, 1994. Current changes in

introducing beneficial microorganisms into the rhizosphere. In: O'Gara F, Dowling DN, Boesten B, eds. *Molecular Ecology of Rhizosphere Microorganisms. Biotechnology and the Release of GMOS*. Weinheim, Germany: VCH, 1–18.

Wulff EG, Mguni CM, Mansfeld-Giese K, Fels J, Lübeck M, Hockenhull J, 2002. Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis*, and *B. pumilis* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. *campestris*. *Plant Pathology* **51**, 574–84.