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Insights on the role played by temperature on the biocontrol agent *Bacillus amyloliquefaciens* strain S499

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Abstract: With the climate change, the temperature is going to play a key role in the effectiveness of microbial biocontrol agents. The influence of this environmental factor on the ecology and biocontrol activity of *Bacillus amyloliquefaciens* strain S499 has been investigated in this work. On this purpose, the effect of temperature on the ability to move onto solid surface, to form biofilm, to persist onto plant rhizosphere and to induce systemic resistance in plants have been evaluated *in vitro* and *in vivo*.

Key words: *Bacillus amyloliquefaciens*, temperature, motility, biofilm, induced systemic resistance

Introduction

Antagonist aptitudes of biocontrol *Bacillus* spp. strains are commonly evaluated under conditions that are favorable to plants and/or microorganisms. The new climate scenarios make necessary to understand how environmental factors can affect the biocontrol effectiveness of a bacterial strain. Temperature is one of the parameters that needs to be taken into account since it plays an important role in the metabolism and physiology of *Bacillus* spp. (Shin *et al*., 2007).

B. amyloliquefaciens strain S499 has shown the ability to protect plants against several phytopathogenic fungi (Jacques *et al*., 1999; Ongena *et al*., 2005). The biocontrol potential of strain S499 mainly relies on the production of lipopeptides of the surfactin, iturin and fengycin families (Ongena & Jacques, 2008). These amphiphilic cyclic peptides are involved in important processes such as biofilm formation, bacterial motility and root colonization (Ongena & Jacques, 2008). Moreover, surfactin can directly interact with plant cells stimulating the induced systemic resistance (ISR) (Henry *et al*., 2011).

Data regarding the regulation of lipopetide production by temperature has already been reported in *B. subtilis* species. In strain RB14, the optimal temperature for the production of iturin was 25°C while that for surfactin was 37°C (Ohno *et al*., 1995). Recently, Fickers *et al*. (2008) showed that the production of the lipopeptide mycosubtilin is affected by temperature and a 30-fold increase is achieved when the temperature decreases from 37 to 25°C.

The aim of the present work was to understand how temperature may modulate some characteristics associated with lipopeptide production. Therefore, the effect of temperature on the motility, the biofilm formation and the capability to protect plants of *B. amyloliquefaciens* S499 was evaluated.

Material and methods

Plants and microorganisms
Strain S499 was routinely grown at 28°C onto Luria-Bertani agar (LBA). Bean (cv. Borlotto), tomato (cv. Tondo rosso) and zucchini (cv. Xara) plants have been used in all experiments. *Botrytis cinerea*, *Phytophthora infestans* and a population of *Podosphaera xanthii* were maintained onto potato dextrose agar, pea agar medium and zucchini plants, respectively. Fresh conidia and sporangia collected either from sporulating colonies or infected leaves were used as inoculum in greenhouse trials.

Motility assays
Strain S499, grown for 72h onto LBA plates at 28°C, was used to produce a solution with a final concentration of 1×10^7 cfu/ml. A volume of 2.5µl of S499 suspension was spotted onto LB containing 0.3 and 0.5% of agar, in order to evaluate swimming and swarming motility, respectively. Once inoculated, plates were incubated at 15, 25, 30 and 35°C and the diameter of bacterial displacement from the point of inoculation was scored after 12 and 24h. Three plates have been inoculated for each temperature and experiments have been carried out three times.

Biofilm formation
Biofilm production assays were carried out in 24-wells polystyrene plates. Half of the wells, containing 1ml of LB, were inoculated with 10µl of a S499 solution (1×10^7 cfu/ml) and incubated for 70h at 15, 25, 30 and 35°C. After the incubation period, the absorbance at 600nm was recorded and all the plates were treated in the same way. Bacterial cultures were removed from the wells and plates were kept at 37°C for 15 min. Cells were fixed to the walls by adding 1.25ml of methanol, incubated at room temperature (RT) for 1 min and then discarded. Wells were washed with 1.25ml of sterile distilled water and dried again at 37°C for 20 min. Adherent cells were stained by adding 1.25ml of crystal violet per wells and incubated for 1 min at RT. The excess stain was removed by inversion of the plate followed by two washings (2.5ml/well each) with sterile distilled water. An ethanol:acetone (4:1) solution was added to wells (2ml/well) in order to decolorize the cells. After 5 min of incubation at RT, the absorbance at OD500 nm was measured for each well.

Greenhouse trials
ISR assays were carried out according to the procedure described by Ongena *et al.* (2002) with some modifications. Bean, tomato and zucchini plants were grown in a sterilized peat substrate. When four leaves were produced, half of the plants were inoculated with 20ml of S499 suspension (1×10^7 cfu/ml) as a drench to roots while the second half was treated with water. Subsequently, all the plants were exposed for seven days at three different temperatures (15, 25 and 35°C). Five replicates (three plants each for plant crops) were used for each temperature. Experiments were carried out twice.

Seven days after the S499 inoculation, each crop was inoculated with the respective pathogen. The inoculation conditions were 90 ± 5% relative humidity and 20°C for 24 hours. Bean leaves were gently wounded with a sterile needle and inoculated by spraying the leaves with a water suspension of *B. cinerea* conidia (1×10^7/ml) plus 0.1% glucose. Six wounds were made on each leaf. Tomato leaves were inoculated by spraying the entire plant with a water suspension of *P. infestans* (1×10^6 sporangia/ml). *P. xanthii* (5×10^7 conidia/ml) were quickly washed from sporulating leaves and immediately sprayed on leaves.

On each plant, the level of disease was expressed in terms of disease severity (percentage of leaf showing disease symptoms) after four days of incubation. During ISR assay, S499 rhizosphere population was evaluated by dilution plating at 2, 5 and 10 days post inoculation (dpi). Results were analyzed using ANOVA and the significance of differences was evaluated by Tukey's test.

Results and discussion

Temperature affects motility and biofilm formation in* Bacillus amyloliquefaciens *S499

Swimming and swarming motility of strain S499 were influenced by temperatures. The higher temperature (35°C) determined a faster motility and S499 macrocolony reached the maximum diameter (85mm) after 12h. On the contrary, S499 motility was drastically impaired when it was incubated at 15°C. In this case, the maximum diameter reached by S499 macrocolony was 7.71 ± 0.39mm and 5.50 ± 0.34mm for swimming and swarming, respectively.

Temperature influenced also the ability to form biofilm. The growth of S499 (OD_{600}) and the quantity of adherent cells (OD_{500}) at 15°C was reduced in comparison with the values observed at the other temperatures. The highest value of biofilm formation was registered at 25°C. Interestingly, temperature influenced the structure of biofilm and pellicles at air-liquid interface developed when the bacterium was incubated at 35°C only.

Influence of temperature on the ability of S499 to colonize plant rhizosphere and induce systemic resistance in plant

B. amyloliquefaciens strain S499 was added as drench to the roots of bean, tomato and zucchini plants subsequently exposed to 35, 25 and 15°C. After seven days, the areal parts of the plants were inoculated with their respective plant pathogen. No differences in plant protection effectiveness of S499 were observed in the pathosystem zucchini x *P. xanthii*, where the disease severity was reduced by ≈ 35%. On the contrary, temperatures affected the plant protection effectiveness of S499 in the bean x *B. cinerea* pathosystem. In this case, the bacterium was able to drastically reduce the disease severity of the plant pathogen when plants were kept at 35°C with a reduction of ≈ 80% of disease severity. Strain S499 determined a less significant decrease of disease severity (42 ± 3%) when bean plants were maintained at 25°C.

However, the application of S499 to the roots gave a generally appreciable reduction of disease severity in all the conditions assayed, with the only exception of the pathosystem tomato × *P. infestans* when plants were kept at 25°C. In this case, the application of S499 did not guarantee any protection towards *P. infestans*. Noteworthy, this environmental condition determined also slower root colonization by S499 in comparison with the other temperatures. Indeed at 10 dpi, a tenfold difference in S499 concentration was observed between plant grown at 25 and 35°C. The higher cell density registered onto tomato roots grown at 35°C determined a reduction of 46 ± 3% of disease severity.

Data reported in this work outline the implication that the changing temperatures can have on the effectiveness of a biological control agent such as *B. amyloliquefaciens* strain S499.

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