Effects of temperature on the protocooperation between plants and *Bacillus amyloliquefaciens* S499

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Abstract: When applied in open fields, the efficacy of biological control agents may be strongly affected by environmental factors such as temperature and water availability. The protocooperation between *Bacillus amyloliquefaciens* S499 and plants has been chosen as a model to investigate how temperature and water regime may modulate the beneficial interaction between the two bionts. On this purpose, the effect of temperature on the ability of S499 to induce systemic resistance and to produce surfactin *in planta* has been investigated in this work.

Key words: *Bacillus amyloliquefaciens*, temperature, surfactin, induced systemic resistance

Introduction

The world is facing considerable climate changes mostly due to the increase of atmospheric CO_2 coming from natural sources or anthropogenic activities (IPCC Climate Change, 2007). As consequence, it is highly probable that growth and antagonistic activity of biological control agents (BCAs) will be affected by these climate changes. Nonetheless, the effect of temperature and water/osmotic stress on several biocontrol mechanisms have been addressed in a few studies (Egamberdijeva & Hoflich, 2003).

Members of the *Bacillus* genus are some of the beneficial microbes most widely used as BCAs and their potential relies on the ability to compete for nutrients, to directly inhibit plant pathogens by producing antibiotic molecules and, in some cases, to stimulate the induced systemic resistance (ISR) in plants (Bakker *et al.*, 2007).

Bacillus amyloliquefaciens strain S499 protects plants against several phytopathogenic fungi by producing lipopeptides of the surfactin, iturin and fengycin families (Ongena *et al.*, 2005 a, b). These amphiphilic cyclic peptides are involved in important processes such as bacterial motility, biofilm formation and root colonization (Ongena & Jacques, 2008). Moreover, surfactin directly interacts with plant cells triggering ISR (Henry *et al.*, 2011).

In this context, the first aim of the present work was to evaluate the effect of low and high temperatures either combined with or without water stress on the potential of *B. amyloliquefaciens* S499 to colonize plant roots and to stimulate ISR. Furthermore, part of the work has been focused on the role played by the temperature on production of surfactin in *B. amyloliquefaciens* S499 both *in vitro* and *in planta*, and how its production affects ISR levels in plants.

Material and methods

Plants and microorganisms

Strain S499 was routinely grown at 28 °C onto Luria-Bertani agar. Bean (cv. Borlotto), tomato (cv. Tondo rosso) and zucchini (cv. Xara) plants have been used in all experiments. *Botrytis cinerea*, *Phytophthora* (*Ph.*) *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.

Greenhouse trials

Two ISR experiments (named Exp. 1 and Exp. 2) were carried out according to the procedure described by Ongena *et al.* (2002) with some modifications. In both the cases, bean, tomato and zucchini plants were grown in a sterilized peat substrate and half of the plants were inoculated with 20 ml of S499 suspension (1×10^7 cfu/ml) as a drench to roots while the second half was treated with water. In Exp. 1, all inoculated and not inoculated plants were exposed for seven days at three different temperatures (15, 25 and 35 °C) combined or not with low water regime. In Exp. 2, S499 inoculation followed the exposure period to different temperatures and water regime. In each case, five replicates (three plants each for plant crops) were used for each temperature and water regime. Experiments were carried out twice.

Seven (Exp. 1) and two (Exp. 2) 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 h. Bean leaves were gently wounded with a sterile needle and inoculated by spraying the leaves with a water suspension of *B. cinerea* conidia (1×10^5/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 *Ph. infestans* (1×10^6 sporangia/ml). *P. xanthii* (5×10^5 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. Results were analyzed using ANOVA and the significance of differences was evaluated by Tukey's test.

Quantification of surfactin in vitro *and* in planta

During this work, the effect of temperature on the *in vitro* production of surfactin by S499 has been determined according to Jourdan *et al.* (2009). S499 was grown for 72 h in Optimal Medium at different temperatures (15, 20, 30, 40 and 48 °C) and supernatants were analysed by HPLC.

Lipopeptides were extracted from the rhizosphere of tomato plants grown in gnobiotic conditions at different temperatures (15, 25 and 35 °C) by adding 6 ml of acetonitrile (ACN)/formic acid 0.1% and 2 g of beads to each tube. Once the supernatants were dried, residues were resuspended in 500 µl of ACN 80%/formic acid 0.1% and processed for analysis of surfactin content by LC-ESI MS. Each combination has been repeated three times in each experiment and two independent experiments were carried out.

In hydroponic cultures, germinated bean, tomato and zucchini seeds were transferred into sterilized 1 l boxes filled with nutrient solution, four plants per box and mantained at 15, 20, 28 and 35 °C. Four boxes of each plant species were grown at each temperature condition with a 16 h photoperiod alternating sunlight and fluorescent light. A volume of S499 cell suspension was inoculated for each box in order to reach a final concentration of 5×10^7

cells/ml in the nutrient solution. Surfactin production on roots was assessed according to the procedure reported by Nihorimbere *et al.* (2012).

Results and discussion

Effects of temperature and water stress on the ability of B. amyloliquefaciens *S499 to trigger ISR*

Root treatment with *B. amyloliquefaciens* S499 significantly reduced disease severity in all conditions tested in both experiments. In Exp. 1, high level of protection was observed in the bean/*B. cinerea* and tomato/*Ph. infestans* pathosystems, while *P. xanthii* infection on zucchini appeared to be less efficiently controlled by S499-triggered ISR. The high rate of disease reduction in bean and tomato is consistent with previous data showing that the strain can readily stimulate some defense mechanisms in the infected tissues of these two host plants (Ongena *et al.*, 2005a, b)

The highest protection levels (up to 65%) were observed on bacterized bean and tomato plants grown at 15 and 35 °C. A consistently lower level of disease control (about 40%) under incubation at 25 °C was provided by S499 in these two crops. These differences in ISR levels probably reflect changes in the activity of S499 cells, given that there was no decrease in ISR efficacy at 25 °C in tomato and bean in Exp. 2 when S499 was applied in soil after the exposure to different temperature/water regimes for seven days.

Moreover, in Exp. 2, simultaneous exposure to 35 °C and water stress led to a reduction in plant protection as a result of ISR triggered by S499 in the three crops probably because temperature and drought may decrease the effectiveness of plant defense mechanisms against biotic factors (Wang *et al.*, 2009). Interestingly, in Exp. 1 no differences in the efficacy of plant protection were observed between plants normally watered and those submitted to drought at any of the tested temperatures. This suggests that the decrease in ISR efficiency due to water stress can be relieved by application of *B. amyloliquefaciens* S499.

Temperature modulates surfactin production in vitro *and* in planta

The exposure of S499 to low temperatures determined an increase in surfactin relative production *in vitro*. Interestingly, the lipopeptide production is clearly impaired at higher temperatures. The data obtained from experiments carried out *in vitro* were confirmed by measuring surfactin quantities produced under gnotobiotic conditions. In fact, the lipopeptide was detected at rates of 1800, 16 and 4 µg /10^8 cfu per g of root in plants grown at 15, 25 and 35 °C, respectively. In these experiments, surfactin was produced at a final concentration of 2-3 µM in the tomato rhizosphere. Interestingly, such a concentration level is biologically relevant, given that pure surfactin stimulates plant defense when added in micromolar quantities (2-10 µM) (Ongena *et al.*, 2007; Jourdan *et al.*, 2009).

In hydroponic experiments, the quantities of surfactin produced at 15 and 35 °C on the roots of tomato and bean plants were higher than at 20 and 28 °C. This fits well with the more highly efficient ISR-based plant protection observed at these temperatures than at 25 °C found in Exp. 1 and clearly supports the involvement of surfactin in ISR mediated by *B. amyloliquefaciens* S499 in these two host plants (Ongena *et al.*, 2007).

This work represents a first step in understanding how different temperatures may affect the interaction between plants and biocontrol agents. In the case of *B. amyloliquefaciens* S499, it seems that both high and low temperatures are not detrimental for surfactin production on plant roots and, as a consequence, the ability to trigger ISR in plants remains well conserved.

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