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Effect of temperature, pH and substrate composition on production of lipopeptides by *Bacillus amyloliquefaciens* 629

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The lipopeptides surfactin, fengycin and iturin produced by *Bacillus* species have diverse ecological roles, including antimicrobial activity, induced systemic resistance in plants against pathogens and plant colonization by the producing strain. The conditions that govern both lipopeptide production and plant colonization are not fully understood. The present study investigated the role of growth media, temperature and pH on the production of the lipopeptides surfactin, fengycin and iturin by *Bacillus amyloliquefaciens* 629 and its production in bean plants colonized epiphytically and endophytically by this isolate. Surfactin was produced at higher amounts when isolate 629 was grown at 15 than at 25 and 30ºC, whereas fengycin remained approximately constant across different temperatures. Iturin was detected on bean stem sap, root exudates and in potato dextrose broth, indicating that plant-derived nutrients play an important role in its production by isolate 629. *B. amyloliquefaciens* 629 colonized plants with more efficacy at 28 than at 20°C. None of the lipopeptides was detected in plants colonized by isolate 629, despite the number of attempts performed with ultra-performance liquid chromatography (UPLC) analysis.

Key words: Bioaccumulation, fengycin, iturin, surfactin.

INTRODUCTION

Lipopeptides are surface-active molecules that may possess antimicrobial activity, induce plant immune responses and may facilitate plant colonization. These compounds play a role in the ecological fitness of the producing organism (Yoshida et al., 2001; Hsieh et al., 2008; Richardson et al., 2009; Raaijmakers et al., 2010;

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Cawoy et al., 2015). Among many active compounds produced by *Bacillus* spp., surfactins, fengycins and iturins are the most frequently reported lipopeptides with a role in biological control of phytopathogens. Surfactins, fengycins and iturins are families of compounds containing chemical variants of each of these lipopeptides (Ongena and Jacques, 2008). Surfactins are bioemulsifiers that promote increased surface area for hydrophobic water-insoluble growth substrates; bioavailability of hydrophobic substrates by increasing solubility; and attachment and detachment of bacteria to and from surfaces (Rosenberg and Ron, 1999). Fengycin and iturins have mostly an antimicrobial role, mainly in the displacement of filamentous fungi and some groups of bacteria from substrates (Vanittanakom et al., 1986, Steller et al., 1999). Lipopeptides play an important role in the modes of action employed by *Bacillus* in the protection of plants against diseases (Raaijmakers et al., 2010). Synergistic effects of surfactins, fengycins and iturins in the control of phytopathogens were reported earlier by Maget-Dana et al. (1992), Ongena et al. (2007) and Romero et al. (2007).

Production of lipopeptides is regulated by a two-component system in a quorum-sensing dependent manner (Duitman et al., 2007). Surfactins are synthesized during the transition from the exponential to the stationary bacterial growth phase, whereas the biosynthesis of fengycins and iturins occurs later in the stationary phase (Vater et al., 2002). Some abiotic factors were shown to interfere with lipopeptide production by *B. subtilis* (Mizumoto and Shoda, 2007; Vater et al., 2002).

Plants interact with microorganisms that surround their tissues and the root system has the strongest influence on this interaction. Plant roots release sugars, amino acids, organic acids and inorganic ions that support the growth and production of bioactive molecules. For instance, *B. amyloliquefaciens* strain S499 produces surfactin, fengycin and iturin in tomato root exudates (Nihorimbere et al., 2012).

Once colonized by beneficial bacteria, plant roots experience long-term benefits, such as increased growth, increased resistance to chemical and physical damage, and direct antagonism against harmful plant pathogens (Punja, 2001; Yoshida et al., 2001; Richardson et al., 2009). Colonization is the first step and a prerequisite for the successful delivery of these benefits to host plants (Steenhoudt and Vanderleyden, 2000). Little is known, however, on the influence of lipopeptides on plant colonization.

Since lipopeptides play a crucial role in the ecological fitness of *Bacillus* species, the objectives of this work were to study: 1) the lipopeptide production in different substrates (MOLP, LURIA, PDB and MB1) and temperatures (30, 25 and 15°C) by *B. amyloliquefaciens* 629; 2) the influence of the substrate pH in lipopeptide production; 3) lipopeptide production using stem sap and root exudates as a sole source of nutrients and growth factors; 4) the endophytic and epiphytic bean plant colonization at 28 and 20°C and the quantification of lipopeptides *in planta*.

MATERIALS AND METHODS

Bacterial isolate and growth conditions

Bacillus isolate 629 was isolated from a healthy adult *Theobroma cacao* tree (Leite et al., 2013). Its identity was confirmed as *B. amyloliquefaciens* on the basis of 16S rDNA (JQ435867), *gyr*A (LN555733) and *rec*A sequences (LN555734). The isolate was deposited in the Biological Institute Culture Collection of Phytopathogenic Bacteria - IBSBF (Campinas, São Paulo, Brazil) under accession number IBSBF-3106. This collection was registered with the World Data Centre for Microorganisms collection under number WDCM-110. A spontaneous rifampicin-resistant (rif^R) variant of 629 able to grow on 100 µg/mL of this antibiotic was used in all experiments. Isolate 629 was grown on liquid MOLP, MB1, Luria-Bertani Broth and PDB at 15, 25 and 30°C for 48 h. The media composition were as follows: MOLP medium (casein peptone 30 g/L, saccharose 20 g/L, yeast extract 7 g/L, KH_2PO_4 1.9 g/L, $MgSO_4$ 0.45 g/L, citric acid 10 mg/L, $CuSO_4$ 0.001 mg, $FeCl_3.6H_2O$ 0.005 mg, $NaMoO_4$ 0.004 mg, KCl 0.002 mg, $MnSO_4.H_2O$ 3.6 mg, $ZnSO_4.7H_2O$ 0.014 mg and H_3BO_3 0.01 mg; pH was adjusted to 7 with KOH) (Ahimou et al., 2000); MB1 liquid (sucrose 10 g/L, casein peptone 8 g/L, yeast extract 4 g/L, K_2HPO_4 2 g/L, $MgSO_4.7H_2O$ 0.3 g/L) (Kado and Heskett, 1970); modified Luria-Bertani Broth (casein peptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L and glucose 1 g/L) (Miller, 1972); and PDB (potato dextrose broth 24 g - Difco). Bacterial suspensions were prepared by growing isolate 629 for 24 h in liquid MB1 medium at 30°C under 120 rpm and adjusted to concentrations that depended on the experiment. The bacterial isolate was stored in 40% glycerol at -80°C.

Influence of culture media, temperature and pH on lipopeptide production

Lipopeptide concentrations were studied in the four different culture media described above and at three temperatures. Erlenmeyer flasks (125 mL holding capacity) containing 25 mL of each culture medium received 50 µL of a *B. amyloliquefaciens* 629 suspension at $2x10^5$ CFU/mL. The treatments with 3 replicates were placed in an orbital shaker at 120 rpm and 15, 25 and 30°C and incubated for 72 h. In PDB medium at 15°C, the bacterium was incubated at up to five days. All experiments were done twice.

To study the production of lipopeptides in different pHs, the MB1 was arbitrarily chosen and 100 µL of a suspension of isolate 629 containing $2x10^5$ CFU/mL was transferred to Erlenmeyer flasks containing 50 mL of medium adjusted to pH 5, 6, 7, 8 and 9. The flasks were incubated in an orbital shaker at 120 rpm at 30°C for 72 h and at the end of the experiment the final pH was measured. Experiments were installed in a completely randomized design with 3 replicates and were done twice.

Extraction and detection of lipopeptides produced *in vitro* was performed by collecting samples of 12 mL from the experiments to analyze medium composition and pH, centrifuged for 10 min at 25,155 g and 10 mL of supernatant was passed through C18 solid-phase extraction cartridges (Grace Maxi-CleanTM SPE 300mg, Alltech Associates Inc., Lokeren, Belgium), previously rinsed with 20 mL methanol and 15 mL MilliQ water, followed by a wash with 5 mL MilliQ water. After this, the lipopeptides adhering to the column were released by passing 1 mL of methanol through the cartridge and transferred to 1.5 mL microcentrifuge tubes. The samples were centrifuged again for 10 min at 25,155 *g* and 300 µL were transferred

to a special tube for ultra-performance liquid chromatography - UPLC analysis (Waters Acquity H-class). The UPLC was coupled to a single mass spectrometer (Waters Single Quad Detector - SQD mass analyzer). UPLC analysis was carried out on a reverse phase column (Acquity UPLC BEH C18 1.7 µm, 2.1 X 50 mm, Waters). Elution was performed at 40°C with a constant flow rate of 0.6 mL/min using a gradient of acetonitrile in water acidified with formic acid (0.1%) as follows: 30% acetonitrile for 2.43 min, from 30 to 95% for 5.2 min, then the column was stabilized at 30% acetonitrile for 1.7 min. The lipopeptides were detected in electrospray positive ion mode by setting the SQD parameters as follows: source temperature, 130°C; desolvation temperature, 400°C; and nitrogen flow, 1000 $L.h^{-1}$. A cone voltage of 120 V was used (Cawoy et al., 2015). After the analyses, the lipopeptides were identified in the chromatogram and spectrogram by mass comparison and retention time.

Production of lipopeptides in bean stem sap and root exudates

Common bean seeds (*Phaseolus vulgaris* - cultivar Pérola) were sown in vermiculite and incubated for 10 days in a chamber adjusted to 28°C. The stems were separated from the leaves and roots, surface-disinfested with alcohol (70% v/v) for 30 s, sodium hypochlorite (2% v/v) for 1 min, and washed three times with sterile distilled water. Forty stems (7.83 g) were transferred to a 1-L beaker with 600 mL of sterile distillated water and incubated at 28°C for 48 h under sterile conditions to extract the stem sap, which was later passed through a 0.22 µm diameter filter to eliminate stem residues. To obtain root exudates, roots of 40 plants (37.56 g) were surface-sterilized, incubated, and filtered as described for the stem sap. Aliquots of 20 mL of the stem or root exudates were transferred to Erlenmeyer flasks (125 mL), and 100 µL of the bacterial suspension ($2x10^5$ CFU/mL) was added to each of the three flasks that constituted the replicates of the experiment that was performed twice in a completely random design. The samples were incubated in an orbital shaker at 120 rpm and 30°C for 120 h, then lipopeptides were analyzed as described above.

Plant colonization and lipopeptide production

These experiments were performed in sterile glass bottles filled with vermiculite and Hoagland's solution was used as the nutrient and water source (Hoagland and Arnon, 1950). Bean seeds were pre-germinated in the vermiculite for 5 days and inoculated by dipping the roots for 30 min in a suspension containing $2x10^5$ CFU/mL. Subsequently, the inoculated plants were incubated at 28°C or 20°C with a 12 h photoperiod for 10 days. Endophytic and epiphytic populations of isolate 629 in the same plant were assessed separately on solid MB1 supplemented with rifampicin. To assess endophytic population, roots, stems and leaves were separated, weighted, surface-sterilized, ground in a mortar and pestle and 10x step dilutions prepared in 0.5% saline solution were plated on MB1 and incubated for 24 h at 30°C. Only samples that showed no bacterial growth after plating aliquots from the third rinse with distilled water during the surface sterilization were used for estimating bacterial population. This procedure was adopted to confirm the endophytic nature of the bacteria from these plant parts. To estimate the epiphytic populations, each plant part was immersed in 0.5% saline solution, vortexed for 1.5 min and 10x step dilutions were plated on MB1 as described above. Extraction and detection of lipopeptides produced *in vivo* was performed by collecting 10-day old bean plants, separating roots, stems and leaves and grinding in a mortar and pestle with liquid nitrogen. Samples of 1 g of the resulting powder were mixed with 10 mL of extracting solution, composed of acetonitrile and 1% formic acid. Samples were dried in a SpeedVac and resuspended in 1 mL of extracting solution. Analysis of the lipopeptides was performed in an UPLC, as described above. The experiments were installed in a completely randomized design with three replicates and performed twice.

Statistical analysis

All statistical analyses were performed with the R software (R Core Team, 2014). Comparisons between the means were performed using Tukey or the t-test for paired data at 5% probability.

RESULTS

Among the different culture media tested, only PDB allowed production of all three lipopeptides at 30 and 25°C, and it was the only medium where iturin was produced, however, there was no bacterial growth at 15°C. In PDB, the production of fengycin and surfactin was the lowest as compared to the production in the other media. Production of surfactin increased as the temperature decreased for all media, except for PDB, whereas fengycin remained at an approximately constant level as the temperatures changed, but with a higher production at 25°C. In addition, the effect of the temperature was most pronounced for surfactin that had its production increased by lower temperatures (Table 1). A representative chromatogram and spectrogram shows the production of the three families of the lipopeptides detected for strain 629 (Figure 1).

The final pH of the medium adjusted to pH 5 to 7 remained similar and for the ones with initial pH of 8 and 9 were lowered to values around 7. Surfactin and fengycin were only detected at initial pH 6 and 7, with the maximum production at pH 6 and iturin was not produced at the same pH (Table 2). Bacterial population sizes did not seem to have influenced the production of lipopeptides and the variation between the lowest and the highest densities was 6.5x at the different pH levels. Stem sap allowed the production of higher amounts of lipopeptides than root exudates. Root exudates showed no production of fengycin and lower populations of bacteria than stem sap (Table 3).

Isolate 629 colonized bean plants epiphytically and endophytically and was recovered from all plant parts, including leaves, stems, and roots when plants were grown at 28°C, whereas no bacteria were recovered from leaves at 20°C. Population densities were similar among all treatments, except for leaves at 20°C (Figure 2). The lipopeptides fengycin, iturin and surfactin were not found in bean plants, irrespective of the incubation temperature and part of the plant analyzed.

DISCUSSION

B. amyloliquefaciens 629 has been reported to promote

Table 1. Detection of lipopeptide production by *B. amyloliquefasciens* 629 grown in different liquid media and incubated at three different temperatures with 120 rpm of shaking for 72 h. Lipopeptides were determined by ultra-performance liquid chromatography (UPLC) analysis.

Culture media	Temperature (°C)	Population (CFU/mL)	µg lipopeptide/mL		
			Surfactin	Fengycin	Iturin
MOPL	30	$1.5 \times 10^7 \pm 6.47 \times 10^6$**	2.92 ± 0.30^{cd}	0.82 ± 0.04^{bcd}	nd*
	25	$8.9 \times 10^6 \pm 5.64 \times 10^6$	4.51 ± 0.97^{c}	1.44 ± 0.73^{abcd}	nd
	15	$3.1 \times 10^7 \pm 2.38 \times 10^6$	9.45 ± 0.81^{b}	0.29 ± 0.10^{d}	nd
MB1	30	$9.1 \times 10^6 \pm 1.29 \times 10^6$	0.37 ± 0.03^{e}	0.44 ± 0.09^{cd}	nd
	25	$7.8 \times 10^6 \pm 1.92 \times 10^6$	5.21 ± 0.07^{c}	1.96 ± 0.04^{ab}	nd
	15	$1.2 \times 10^7 \pm 2.66 \times 10^6$	14.39 ± 2.32^{a}	1.86 ± 0.51^{abc}	nd
PDB	30	$4.9 \times 10^6 \pm 1.14 \times 10^6$	0.30 ± 0.01^{e}	0.14 ± 0.05^{d}	0.72 ± 0.14^{a}
	25	$1.1 \times 10^7 \pm 1.95 \times 10^6$	0.44 ± 0.03^{e}	0.24 ± 0.07^{d}	3.05 ± 0.25^{b}
	15	nd	nd	nd	nd
Luria	30	$6.8 \times 10^6 \pm 3.72 \times 10^6$	1.91 ± 0.88^{de}	2.04 ± 1.93^{ab}	nd
	25	$3.5 \times 10^6 \pm 1.90 \times 10^6$	3.76 ± 0.79^{cd}	2.63 ± 0.87^{a}	nd
	15	$7.1 \times 10^6 \pm 2.82 \times 10^6$	10.36 ± 2.46^{b}	0.84 ± 0.70^{bcd}	nd

Means followed by the same letter in the columns are not significantly different according to Tukey's test ($p<0.05$). *nd - not detected. **Standard deviation.

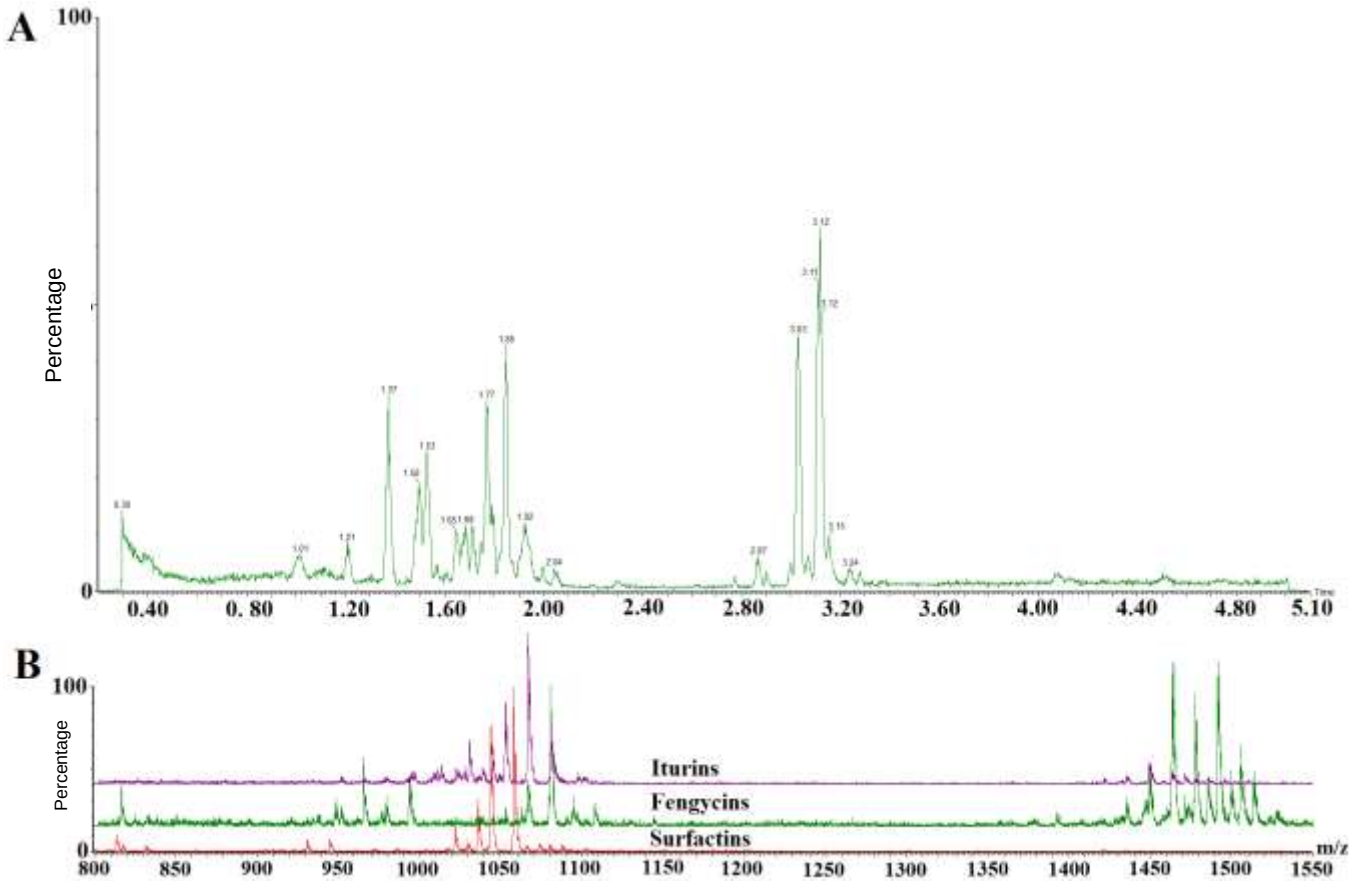

Figure 1. Lipopeptides produced by the strain 629. A representative chromatogram (A) and spectrogram (B) showing iturins, fengycins and surfactins produced by *B. amyloliquefaciens* 629 in PDB at 25°C during 72 h of incubation.

Table 2. Detection of lipopeptide production by *B. amyloliquefasciens* 629 grown in liquid MB1 medium adjusted to different initial pHs and incubated at 30°C with 100 rpm of shaking for 72 h. Lipopeptides were determined by ultra-performance liquid chromatography (UPLC) analysis. Iturin was not detected.

Initial pH	Lipopeptide	µg/ml	Final population (CFU/mL)	Final pH
5	Surfactin	nd*	$1.3 \times 10^5 \pm 1.33 \times 10^3$	5.18±0.18
	Fengycin	nd		
6	Surfactin	4.54±1.81[a]**	$3.8 \times 10^4 \pm 2.21 \times 10^2$	6.28±0.11
	Fengycin	1.72±0.14[a]		
7	Surfactin	0.53±0.02[b]	$2.0 \times 10^4 \pm 1.49 \times 10^3$	6.88±0.07
	Fengycin	0.10±0.01[b]		
8	Surfactin	nd	$5.4 \times 10^4 \pm 4.25 \times 10^4$	6.84±0.17
	Fengycin	nd		
9	Surfactin	nd	$6.3 \times 10^4 \pm 4.46 \times 10^4$	6.88±0.27
	Fengycin	nd		

Means with the same letter are not significantly different according to the t-test ($p<0.05$). Only means of the same lipopeptide in different pH were compared. *nd- not detected. **Standard deviation.

Table 3. Detection of lipopeptide production by *B. amyloliquefasciens* 629 grown in bean stem sap and root exudates and incubated at 30°C with 120 rpm of shaking for 120 h. Lipopeptides were determined by ultra-performance liquid chromatography (UPLC) analysis.

Plant organ	Population (CFU/mL)	Lipopeptides (µg/mL)		
		Surfactin	Fengycin	Iturin
Stem sap	$1.8 \times 10^4 \pm 1.65 \times 10^2$**	0.46±0.21[a]	0.03±0.02	2.23±1.05[a]
Root exudate	$6.7 \times 10^3 \pm 4.03 \times 10^3$	0.03±0.01[b]	nd*	0.08±0.06[b]

Means with the same letter are not significantly different according to the t-test ($p<0.05$). Only means of the same lipopeptide were compared. *nd - not detected. **Standard deviation.

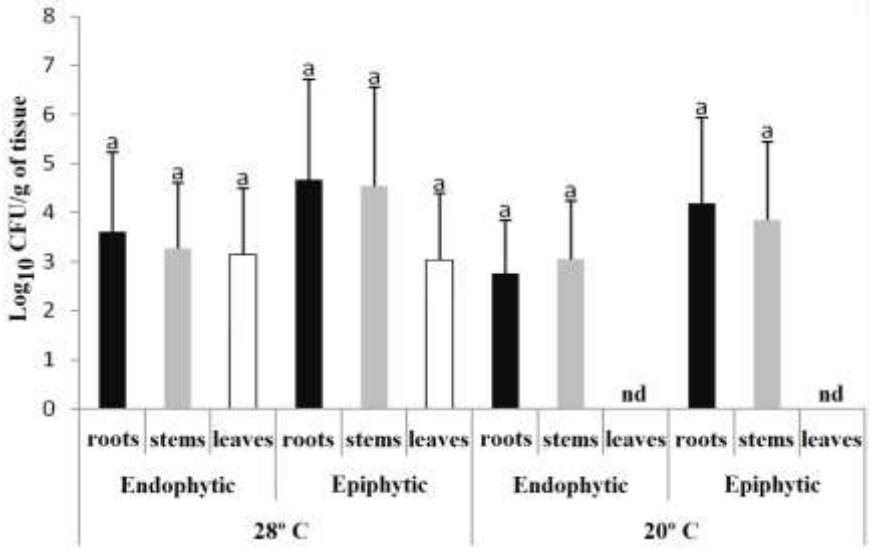

Figure 2. Endophytic and epiphytic colonization of bean plants by *B. amyloliquefaciens* 629. Inoculated plants were cultivated inside glass bottles with vermiculite and incubated at 28 and 20°C for 10 days. Bacterial populations were determined in (endophytic) and on (epiphytic) different plant parts by plating dilutions on solid MB1 medium with rifampicin. Error bars represent the standard error of the means. Means followed by the same letter are not significantly different according to Tukey's test ($p<0.05$). nd- not detected.

cacao growth and to have an antagonistic effect against witches' broom etiologic agent, *Moniliophthora perniciosa* (Falcao et al., 2014), and to reduce the bacterial wilt severity caused by *Curtobacterium flaccumfaciens* pv. *flaccumfacies* (Martins et al., 2013). In addition, strain 629 increases magnesium content in the common bean shoots (Martins et al., 2015).

The lipopeptides fengycin, surfactin and iturin are commonly produced by *Bacillus* species in common culture media (Akpa et al., 2001; Mukherjee and Das, 2005). In this study, it was found that *B. amyloliquefasciens* 629 produces the lipopeptides iturin, fengycin and surfactin in a temperature- and medium composition-dependent manner. In the first step of the experiments, isolate 629 only produced iturin in PDB; although, all tested culture media supported similar bacterial densities. The optimized medium referred to as MOLP (Ahimou et al., 2000) was not the best substrate for lipopeptides production by strain Alb 629.

Surfactin acts as a surfactant and lowers the surface tension to facilitate swarming motility and also was shown to induce systemic resistance (Sachdev and Cameotra, 2013; Cawoy et al., 2014; Phae and Shoda, 1990; Hsieh et al., 2008). Fengycin is also an antimicrobial lipopeptide, but does not have the broad-spectrum activity of iturin (Ongena et al., 2010).

The initial pH close to neutral or slightly acidic favored the production of surfactin and fengycin, coinciding with the optimal pH range for bacterial growth, as demonstrated by Makovitzki and Shai (2005) and Mandal et al. (2013).

Lipopeptides including iturins were also produced in bean stem sap and in root exudates, but they were not detected in bean plants colonized epiphytically and endophytically by isolate 629, even after several attempts. Tissue specific induction of lipopeptide production is likely to occur, since stem sap exudates induced a 15- and 27-fold increase in the concentrations of surfactin and iturin, respectively, while it only promoted a 2-fold increase in bacterial populations. This result suggests that plant-derived factors contribute to the regulation of iturin production by isolate 629, as also observed by Raaijmakers et al. (2010).

Although, surfactin plays a significant role in the colonization of tomato root surfaces (Nihorimbere et al., 2009), it does not seem to be important in the acropetal movement of isolate 629 in bean plants, since isolate 629 was not detected in leaves at 20°C, which is a favorable temperature for the production of the lipopeptide *in vitro*.

It is postulated that either the adopted methods are not suitable for lipopeptide detection *in vivo* or the plant has the ability to interact or modify the molecules, turning them into another compound (Suga and Hirata, 1990). Other plants may induce a differential production of lipopeptides and may be tested in future studies allied with the use of more sensitive techniques such as time of flight – secondary ion mass spectrometry (TOF-SIMS) (Nihorimbere et al., 2012).

Conclusions

Lipopeptides production is both qualitatively and quantitatively affected by differences in substrate, temperature and pH. Surfactin is the most influenced by the temperature, while pH range of 6 and 7 favor the production of fengycin and surfactin. Iturin is only produced in media containing plant-derived nutrients. Although, *B. amyloliquefaciens* 629 colonizes plants endophytically and epiphytically, none of the lipopeptides were detected in bean plants.

Conflict of Interests

The authors have not declared any conflict of interests.

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