J. Microbiol. Biotechnol. (2011), **21**(1), 81–88
doi: 10.4014/jmb.1007.07047
First published online 11 November 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Evaluation of Anti-Phytoplasma Properties of Surfactin and Tetracycline Towards Lime Witches' Broom Disease Using Real-Time PCR

Askari, N.[1,2], Gh. Salehi Jouzani[1]*, M. Mousivand[1], A. Hagh Nazari[2], S. Abbasalizadeh[1], and S. Soheilivand[1]

[1]*Microbial Biotechnology and Biosafety Department, Agricultural Biotechnology Research Institute of Iran (ABRII), Mahdasht Road, P.O. Box 31535-1897, Karaj, Iran*
[2]*Department of Agronomy and Plant Breeding, College of Agriculture, Zanjan University, P.O. Box 45195-313, Zanjan, Iran*

Received: July 24, 2010 / Revised: October 11, 2010 / Accepted: October 12, 2010

The anti-phytoplasma activities of surfactin (derived from Iranian native *Bacillus subtilis* isolates) and tetracycline towards *Candidatus* "Phytoplasma aurantifolia", the agent of lime Witches' broom disease, were investigated. HPLC was used to quantify the surfactin production in four previously characterized native surfactin-producing strains, and the one producing the highest amount of surfactin (about 1,500 mg/l) was selected and cultivated following optimized production and extraction protocols. Different combinations of purified surfactin and commercial tetracycline were injected into artificially phytoplasma-infected Mexican lime seedlings using a syringe injection system. An absolute quantitative real-time PCR system was developed to monitor the phytoplasma population shifts in the lime phloem during 3 months following the injections. The results revealed that the injections of surfactin or tetracycline had a significant inhibitory effect on *Candidatus* "P. aurantifolia". However, the combined treatment with both surfactin and tetracycline (1:1) resulted in the highest inhibition due to a synergic effect, which suppressed the phytoplasma population from about 2×10^5 to less than 10 phytoplasma units/g plant tissue.

Keywords: *Bacillus subtilis*, HPLC, phytoplasma, real-time PCR, surfactin, Witches' broom disease of lime

Phytoplasma-associated diseases are spread worldwide, in several cases being associated with severe epidemics of quarantine importance, and have been associated with diseases from more than 700 plant species, including several horticultural, ornamental, and crop plants [6, 16, 20, 21, 27, 33, 34]. These plant pathogens are prokaryotes belonging to the Mollicutes class, since they lack a cell wall, and up to now they have not been cultivated in an axenic culture. They are absolute parasites of plant phloem and transmitted by insect vectors, especially by leafhoppers [5, 19]. Fortunately, specific primers designed for highly conserved genes, such as the 16S ribosomal gene, together with the use of molecular probes randomly cloned from the phytoplasma genome, allow these pathogens to be discriminated and molecularly classified [2, 4, 6, 17, 21, 27, 33].

The small-fruited lime (*Citrus aurantifolia*) is the most important horticultural plant cultivated in southern regions of Iran. However, Witches' broom disease of lime (WBDL) caused by *Candidatus* Phytoplasma aurantifolia is a serious threat to lime production in the southern parts of Iran and other countries in the region of the Persian Gulf (Alikhani *et al*. 2010. Abstract. 4th ESF Conference on Functional Genomics & Disease, Dresden, Germany, p. 73). The history of the disease dates back to 1986, when it was observed for the first time in Oman [13, 34]. The disease was later reported in the United Arab Emirates [14], India [15], Iran [5], and Saudi Arabia [3]. Currently, there is no efficient control method for WBDL, and therefore, effort is being made to find new anti-phytoplasma agents with novel modes of action [30]. Until now, several antibiotics (tetracycline, oxytetracycline, streptomycin, erythromycin), plant resistance inducers (fosetyl Al and chitosan), and secondary metabolites of fungal (cercosporin, cladosporol, and spirolaxin) and plant origins (pulegone and carvone) have been used against different phytoplasmas [7, 8, 36].

The soil bacterium *Bacillus subtilis* produces different kinds of substances with antibiotic activities. Surfactin is produced by different strains of these bacteria, and is known as one of the most powerful biosurfactants with

*Corresponding author
Phone: +98 (261)2703536; Fax: +98 (261)2704539;
E-mail: gsalehi@abrii.ac.ir

antimicrobial properties [26, 28]. Chemically, surfactin (MW: 1,036 Da) is a cyclic lipopetide consisting of a loop of seven amino acids (LLDLLDL) bound to a β-hydroxy 10- to 13-membered fatty acid chain [18]. Although surfactin was discovered about 40 years ago, there has been a revival of interest in this compound over the past decade, triggered by an increasing demand for effective biosurfactant, antimicrobial, and hemolytic agents. As a result, diverse new properties, including bioremediation [32], antitumoral [24], emulsification, foaming [9], inhibition of starfish oocyte maturation, anti-apoptosis [22], antibiofilm formation [25], and antimycoplasmic activities [30] have recently been demonstrated. Yet, despite the many advantages of surfactin over chemical agents, there are relatively few actual applications of surfactin, mainly due to poor strain productivity and the need for expensive substrates [10, 26].

Therefore, given the above-mentioned properties of surfactin and continuous threat of WBDL epidemics in the region of the Persian Gulf, evaluating the anti-phytoplasma activities of surfactin is very important. Previously, the current authors identified eight surfactin-producing *B. subtilis* strains collected from different ecological zones in Iran using different methodologies, including a blood agar test, *sfp* gene PCR amplification, drop-collapse, and HPLC analyses [26]. The present study investigates the anti-phytoplasma activity of surfactin, alone and in combination with tetracycline as a reference compound, towards the phytoplasma agent of WBDL using a real-time PCR technique for quantitative detection.

Materials and Methods

Bacterial Strains

Four native surfactin-producing isolates of *B. subtilis*, BS119m, BS116l, N3dn, and BS113 [26], were obtained from the Microbial Gene Bank of the Agricultural Biotechnology Research Institute of Iran. The isolates were routinely maintained on a TBAB agar [Difco tryptose blood agar base with 1.5% (w/v) Difco agar; Difco Laboratories Inc., Detroit, MI, USA] and stored frozen in dimethyl sulfoxide [7% (v/v)] at −80°C [26].

Healthy and Infected Lime Plants

Healthy Mexican lime seedlings were kindly provided by the Agricultural Research Centres of Fars and Hormazgan, Iran. Phytoplasma-infected lime plants were obtained by grafting infected seedlings onto healthy lime stems. For this, about fifty 8-month-old Mexican lime seedlings were grafted using twigs taken from an infected 12-year-old lime tree and kept at 25−30°C in a greenhouse. After 4 months, the infected plants exhibited disease symptoms, including broom-like branches and pale leaves, as seen with the source of the disease.

Surfactin Production and Purification

Cultures of the four native *B. subtilis* strains were taken from the −80°C frozen stock and transferred onto an agar medium. Cells from the agar slant were then inoculated into 100 ml of a nutrient broth medium (NB), composed of 3 g/l yeast extract and 5 g/l peptone, in 250-ml flasks and incubated in a gyratory shaker at 200 rpm for 14 h at 30°C. In the second culture stage, 0.5 ml of the seed culture was inoculated into 200 ml of a mineral salt medium (MMS) [31].

The purification of the surfactin was performed according to Abdel-Mawgoud *et al*. [1] with minor modifications. To extract the surfactin, the bacterial cells were removed from the liquid culture by centrifugation at 15,000 ×*g* for 10 min at 10°C. The supernatant was then acidified with 1 N HCl to pH 2 and left overnight at 4°C. Next, the off-white to buff cake produced in the centrifuge tubes was dried in a hot-air oven at 70°C, and the dried materials were transferred to 50 ml of methylene chloride contained in a 250-ml flask and left covered overnight at room temperature with intermittent shaking. The organic extract was then filtered, and the residue on the filter paper re-extracted with 50 ml of fresh methylene chloride and refiltered. The pooled organic phase was evaporated under a vacuum (Buchi, Germany) at 40°C. The residue obtained was then characterized as such or after being dissolved in a 5 mM Tris-HCl buffer, pH 8.5 [1].

Analytical Methods

The concentration of surfactin in the culture supernatant was determined using a reverse-phase HPLC equipped with a Eurospher C18 column according to Mohammadipour *et al*. [26]. To determine the suitable wavelength, the UV spectrum of the sample was determined between 190 and 280 nm using a UV–Vis spectrophotometer (Cary300; Varian, Australia). The surfactin was eluted for 12 min under 90% acetonitrile/10% water/0.1% trifluoroacetic acid (v/v/v) at 0.8 ml/min. The peaks were generated and quantified using a UV detector and ChromGate HPLC software at 205 nm. The quantification of surfactin was inferred from the standard curve of a commercially available surfactin compound (98% pure; Sigma-Aldrich).

Surfactin and Tetracycline Treatments and Injections

To evaluate the anti-phytoplasma activities of surfactin and tetracycline, 4 different treatments were injected into the phloem of the infected seedlings for endotherapeutic intervention of phytoplasma. Four lime plants were assigned to each of the four treatments, which were (i) surfactin (150 mg/l), (ii) surfactin (300 mg/l), (iii) tetracycline (150 mg/l), and (iv) surfactin (150 mg/l)+tetracycline (150 mg/l). An autoclaved 5 mM Tris HCl buffer, pH 8.5, was used as the negative control. The treatments in a volume of 1 l were conducted using a drop injection system once. After the injection, the treated plants were maintained under the same conditions. Leaves were sampled from the treated and control seedlings to measure the phytoplasma population before the injection and 1 week, 2 weeks, 1 month, 2 months, and 3 months after the injection. For each treated plant, leaves were sampled from different places, sampled, homogenized, and used for DNA extraction.

Molecular Detection of Phytoplasma Associated with WBDL

The total genomic DNA was isolated from the healthy and infected lime plants using a CTAB method, as previously described [12]. For the PCR detection of phytoplasma associated with WBDL in the infected seedlings, a two-step nested PCR was performed using two pairs of primers: universal primers p1/p7 [11] and specific primers fe1 (5'-GAGTTAGATAGAGGCGAGTG-3') and re1 (5'-TAATCCT

GTTTGCTCCCCAC-3'). The specific primers fe1/re1 were designed using Oligotech ver. 100 software and GenBank for the specific amplification of a 136 bp fragment in the p1/p7 PCR product from the WBDL. The temperature profile for each PCR consisted of a first denaturation step of 2 min at 94°C, followed by 35 cycles of 1 min/94°C for denaturation, 55°C/1 min for annealing, and 72°C/2 min for extension. A final extension was carried out at 72°C/3 min. The PCR products from the second step were purified using a DNA gel extraction kit #k0513 (Fermentaz, Lithuania) according to the manufacturer's protocol, cloned into a pTZ-Easy T-vector (Fermentaz, Lithuania), and sequenced. The sequences obtained were then aligned and compared with available phytoplasma sequences in the GenBank database using the BLAST search facility at the National Center for Biotechnology Information (NCBI).

Real-Time PCR Primers and Amplification Conditions

Two specific PCR primer pairs, IMP3-F (5'-AGTTGGTGTGTTAGC ATCTTT-3')/IMP3-R (5'-CTACTCTTTGTTTTCCACTT-3') and IMP4-F (5'-AACAAAGCAGATGATAAAGATAA-3')/IMP4-R (5'-TCTTT AGGAGCAGCACTTTCTT-3'), were designed using Oligotech ver. 100 software for conducting the real-time PCR. These primers amplified 158 bp and 118 bp DNA fragments of the gene encoding the immune-dominant membrane protein of WBDL phytoplasma (Accession No. GU339497), respectively. To evaluate the specificity of the primers, a PCR was performed on the infected and healthy plants. The temperature profile for each PCR consisted of a first denaturation step of 2 min at 94°C, followed by 35 cycles of 1 min/94°C for denaturation, 1 min 55°C (IMF3-F/IMF3-R) and 56°C (IMF4-F/IMF4-R) for annealing, and 72°C/2 min for extension. A final extension was carried out at 72°C/3 min. The PCR fragments were cloned into a pTZ-Easy T-vector (Fermentaz, Lithuania) and sequenced. The sequences obtained were then aligned and compared with available phytoplasma sequences in the GenBank database using the BLAST search facility at the NCBI.

The real-time PCR was performed using a MyiQTM Single Color Real Time-PCR Detection System (Bio-RAD, Germany) with an SYBR Green PCR Master Mix (Applied Biosystems) according to Torres *et al*. [29]. The real-time PCR cycling conditions were as follows: 2 min at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 56°C, and finally 30 s at 72°C.

Construction of Artificial Template and Calibration Curve

The PCR primers IMP1-F (5'-CAACGTCGACAAAATCACAAAG AAAATTTTTTAC-3')/IMP1-R (5'-CAACGCGGCCGCTTATGAT AATTTTAAATCTG-3') and IMP1-F/IMP2-R (5'-CAACGCGGCC GCTGATAATTTTAAATCTGATTTAG-3') were used to amplify 540 bp and 537 bp DNA fragments of the gene encoding the immune-dominant membrane protein of the WBDL phytoplasma (also containing IMP3-F/IMP3-R and IMP4-F/IMP4-R), respectively (Accession No. GU339477). The total genomic DNA of the infected lime plants was used as the matrix for the PCR. The amplified fragment obtained from one of the primer pairs was purified using a DNA gel extraction kit #k0513 (Fermentaz, Lithuania) according to the manufacturer's protocol and cloned into a pTZ-Easy T-vector (Fermentaz, Lithuania). The purified plasmid clone was then quantified using a Nano-drop Spectrometer. To construct standards, the artificial template was diluted at final concentrations of 10^7 to 10^1 copies of the amplicon per microliter of sterile water, based on Torres *et al*. [29].

The standards dilutions were then used to establish a calibration curve by plotting the threshold cycles (Ct) obtained based on 40 cycles of the real-time PCR with the IMP3-F/IMP3-R primers versus the $\log_{10}$ of the copy number of the target immune-dominant membrane gene fragment [$\log_{10}$ (copy number)]. Each calibration standard was tested in triplicate or quadruplicate in two different runs. The calibration curve was computed as a linear regression model of the $\log_{10}$ (copy number) as an independent variable for each separate run [29]. All the statistical tests were computed using the statistical packages NTSYS and SPSS ver. 11.

Quantification of Phytoplasma Associated with WBDL in Treated Lime Plants

Dilutions of the artificial template and target treated and untreated plants were amplified based on 40 cycles of a real-time PCR with the IMP3-F/IMP3-R primers during the same reaction. The sample copy number values were then estimated by computing estimates of the linear regression coefficients and the 95% confidence interval of the individual predictions. The data analysis of the real-time PCR was performed using software designed by Bio Rad (Germany) and based on the Ct value. The average Ct value was calculated for each treatment, and the product identity was also confirmed by electrophoresis in a 2% agarose gel. The Ct values were calculated by plotting the normalized fluorescence (ΔRn) in relation to the cycle number. The statistical analysis was performed based on a statistical factorial experiment.

RESULTS

To confirm our previously reported results [26] on the production of surfactin in native strains, four native surfactin-producing *B. subtilis* strains, BS119m, BS116l, N3dn, and BS113, were selected and evaluated using an HPLC analysis. As a result, 8–10 peaks were observed for surfactin extracted from the selected strains, and 9 major peaks, which were similar to those of standard surfactin, were selected through calibration as representing surfactin (4.3–9.0 min retention time). These peaks showed different isomers of surfactin, although the relative abundance of each isomer was not the same for all the samples. As strain Bs119M exhibited the highest surfactin production (1.5–1.6 mg/l culture), it was used for the subsequent surfactin production and purification.

Confirmation of the presence of phytoplasma in the artificially infected lime seedlings was performed based on a two-step nested PCR methodology using universal and specific primers P1/P7 and fe1/re1, respectively. As a result of the first step, a 1.788 bp fragment containing 16S rDNA and a partial sequence of the 23S rDNA gene of phytoplasma was amplified. Meanwhile, in the following step, using an amplicon of the first-step PCR as a matrix, a 136 bp PCR fragment was obtained (Fig. 1). Cloning, sequencing and alignment of the cloned PCR fragment then confirmed the reliability and specificity of the primers and the presence of phytoplasma in the seedlings. Database

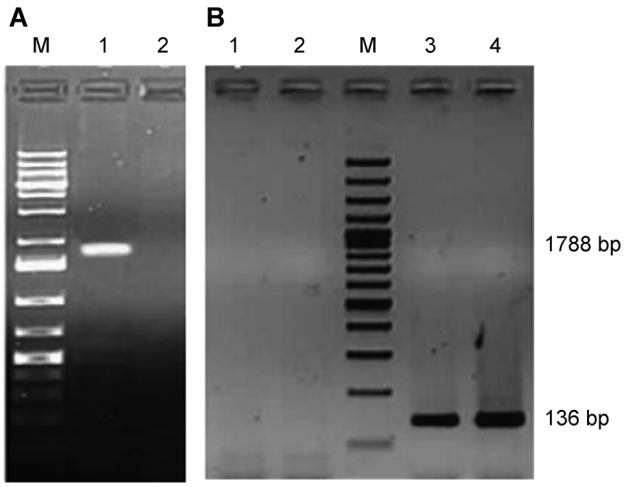

Fig. 1. Direct and nested PCR amplification of WBDL-infected plant genomic DNA using P1/P7 (**A**) and fe1/re1 (**B**) primers.
A. M: 1 kb molecular ladder; 1: WBDL-infected lime plant; 2: Healthy lime plant. **B**. 1: Healthy lime plant; 2: DNA-free reaction; M: 1 kb molecular ladder; 3 and 4: Amplification of first PCR run amplicons from WBDL-infected plant.

blasting revealed that the sequence had a 100% homology with Accession No. U15442 in the NCBI database belonging to the phytoplasmal agent of WBDL. The PCR-positive and morphologically obvious infected seedlings were then used for the subsequent treatments.

To evaluate the anti-phytoplasma activities of surfactin and tetracycline towards the agent of WBDL, the changes in the phytoplasma populations in the plant phloem were used as a criterion of the toxicity of the different treatments. A real-time PCR technique was used to quantify and monitor the changes in the phytoplasma populations in the treated and untreated infected plants. For this purpose, two specific primers amplifying the gene encoding the immune-dominant protein of the cell membranes were designed and synthesized. To evaluate the IMP1-F/IMP1-R, IMP1-F/IMP2-R, IMP3-F/IMP3-R, and IMP4-F/IMP4-R primer specificity, a common PCR was used. All four primer pairs demonstrated specificity for the phytoplasmal agent of WBDL, and the expected fragment sizes were observed, but this was not observed for the healthy plants (Fig. 2). As a result, the primer pairs IMP1-F/IMP1-R and IMP3-F/IMP3-R were used for constructing a standard curve and the real-time PCR reactions, respectively. In the next step, the real-time PCR melting peak was measured. Finally, after a real-time PCR using DNA extracted from the untreated and treated lime plants, a unique melting peak at 77°C (±0.5) was observed, which confirmed the specificity of the IMP3-F/IMP3-R primers (Fig. 3A).

To optimize the specific amplifications, the standards for the partial immune-dominant protein gene were run in a 40-cycle real-time PCR using the IMP3-F/IMP3-R primers. The real-time PCR reactions were also checked using a gel electrophoresis analysis. Only a single band of the expected size (158 bp) was observed for the standard copy numbers and total genomic DNA obtained from the treated and untreated infected plants. Meanwhile, no band

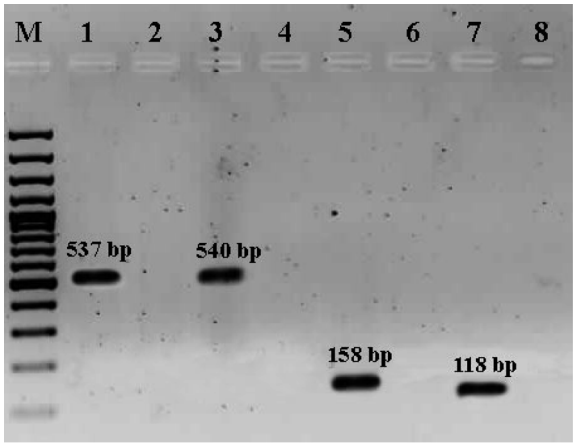

Fig. 2. Evaluation of real-time PCR primer pairs using common PCR.
M: 100 bp molecular ladder; 1–8: Amplified infected and healthy plants genomic DNA using IMP1-F/IMP2-R (1, 2), IMP1-F/IMP1-R (3, 4), IMP3-F/IMP3-R (5, 6), and IMP4-F/IMP4-R (7, 8) primers, respectively.

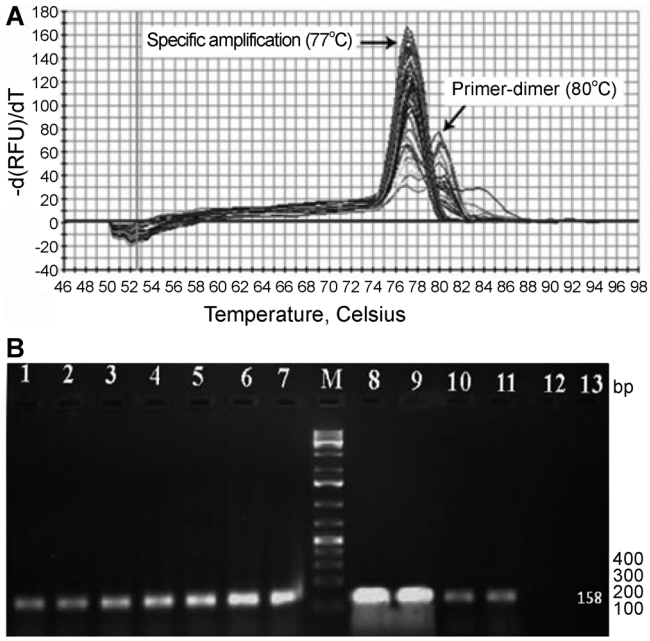

Fig. 3. Specificity of real-time PCR (IMP3-F/IMP3-R) for detection and quantification of phytoplasma from Mexican lime.
A. Measured melting curves. A single peak was observed at 77°C for the treated and untreated infected plants, whereas primer-dimer accumulation was observed for the healthy plants. The DNA-free control did not show any peak. **B**. Agarose gel analysis. A band of the expected size (158 bp) was observed for serial dilutions from 10^1–10^7 (lanes 1–7) for the untreated (lanes 8–9) and treated (lanes 10–11) infected plants. No band was observed for the DNA-free control (lane 12) and healthy plant (13). M: 100 bp molecular ladder.

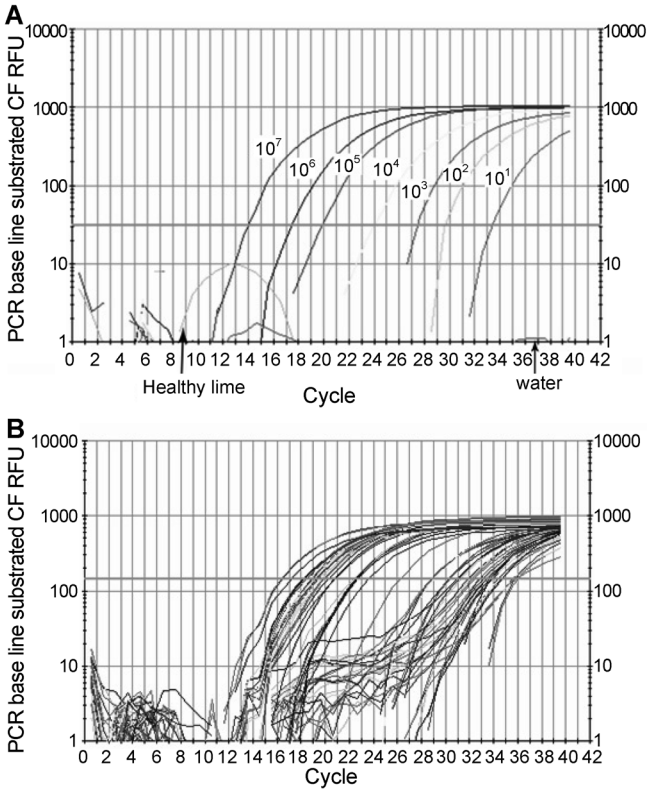

Fig. 4. Evaluation of the sensitivity of the quantitative real-time PCR using standard curve construction (40 cycles).
A. Amplification plot of normalized fluorescence for standards (10^1–10^7 copy number of partial IMP gene fragment) used in the calibration curve.
B. Logarithmic curves obtained by amplification of the partial IMP gene fragment from treated and untreated infected and healthy plants.

was observed for the healthy lime plants and DNA-free controls. It was remarkable that the gel electrophoresis results were able to semiqualitatively differentiate the treatments based on their impact on the phytoplasma populations, as indicated by the sharpness of the observed bands (Fig. 3B).

To construct a standard curve, two different runs were conducted using 10^1 to 10^7 copy number dilutions. Both runs showed a high linear dependence between the two variables, with correlation coefficients of about 0.99. A statistical analysis also confirmed the linearity between 10^1 and 10^7 (Fig. 4A) of the Ct versus the logarithm of the copy number of the partial immune-dominant protein gene. The threshold cycle (Ct) in the real-time PCR reactions was used as a criterion for quantifying the phytoplasma in the treated plants.

A statistical analysis of the results obtained from the treatments showed that all the applied concentrations of surfactin and tetracycline had a significant toxic effect on the phytoplasma populations. Owing to the reduction of the phytoplasma populations, the total DNA of the treated seedlings infected with the phytoplasma associated with WBDL exhibited a significant decrease in fluorescence, in contrast to the untreated plants (controls) when amplified based on a 40-cycle real-time PCR (Table 1). In addition, the mean Ct value for the treated plants significantly increased (average 10 cycles) in comparison with that for the untreated infected plants, also indicating a decrease in the amount of phytoplasma in the treated samples. Overall, the number of phytoplasmas per gram of treated lime seedlings was reduced from $10^5 - 6\times10^5$ to about $10 - 10^3$. From the first week to 12 weeks (3 months) after the injection of surfactin and/or tetracycline into the infected lime seedlings, the number of phytoplasmas continuously decreased. The average increase in the Ct value was 9.4, 9.6, 8.4, and 14 after the injection treatment with tetracycline (150 mg/l), surfactin (150 mg/l), surfactin (300 mg/l), and surfactin (150 mg/l)+tetracycline (150 mg/l, respectively (Table 1). The maximum inhibitory effect was observed three months after injection when using the mixture of both surfactin and tetracycline, where the number of phytoplasmas was reduced to less than 10 per gram of lime seedling tissue. Moreover, in this case, 3 months after

Table 1. Real-time PCR threshold cycles (Ct) and WBDL phytoplasma copy number in infected lime plants after treatment with surfactin and tetracycline.

Time	Before inj.	1 week[a]	2 weeks	4 weeks	8 weeks	12 weeks
Control (without injection)	17.63±0.8[b] (6×10^5)[c]	19.20±1 (3.5×10^5)	19.22±1.1 (3.5×10^5)	18.67±1 (5×10^5)	18.46±0.9 (5.5×10^5)	18.90± 1(a) (5×10^5)
Tetracycline (150 mg/l)	18.50±0.8 (5×10^5)	24.50±1.2 (9×10^3)	23.47±1.3 (2×10^4)	24.39±1.6 (9×10^3)	27.72±1.9 (8×10^2)	27.80±1.5(b) (8×10^2)
Surfactin (150 mg/l)	20.20±1 (10^5)	19.59±1 (2×10^5)	25.22±1.6 (5×10^3)	21.31±1.3 (6×10^4)	26.08±1.7 (3×10^3)	30.00± 2(b) (5×10^2)
Surfactin (300 mg/l)	18.52±0.7 (5×10^5)	20.86±0.9 (7×10^4)	20.48±0.9 (8×10^4)	21.81±1.3 (5×10^4)	22.16±1.5 (4.5×10^4)	26.60±1.8 (b) (10^3)
Surfactin+Tetracycline (each 150 mg/l)	19.60±0.8 (2×10^5)	19.80±0.9 (10^5)	21.52±0.8 (6×10^4)	20.10±1.2 (10^5)	31.56±1.8 (6×10)	34.50±2.2(c) (10)

[a]After injections. [b]Real-time PCR threshold cycles (Ct). [c]WBDL copy number (Approximately).

injection, the infected branches died and new healthy branches appeared that did not exhibit any phytoplasma morphological infection symptoms. Therefore, these results confirmed that surfactin and tetracycline had a synergic effect on the phytoplasma, implying that the simultaneous application of surfactin and tetracycline may be more effective for controlling the disease.

Discussion

Witches' broom disease of lime associated with *Candidatus* P. aurantifolia is a serious problem for lime production in the southern parts of Iran and other countries in the region of the Persian Gulf. However, unfortunately, owing to the regional nature of the disease, there have been no global efforts to control WBDL, to the best of our knowledge, only a few regional studies on efficient control methods [3, 14, 15, 35] (Alikhani *et al*. 2010. Abstract 4th ESF Conference on Functional Genomics & Disease, Dresden, Germany, p. 73). Accordingly, this study was conducted to evaluate the anti-phytoplasma activity of surfactin and tetracycline towards *Candidatus* P. aurantifolia associated with WBDL.

In addition to antivirus, antibacterial and antifungal activities, surfactin has also been shown to possess antimycoplasma activity [25, 30]. Mycoplasmas similar to phytoplasmas belong to the class Mollicutes and lack cell walls, but in contrast to phytoplasmas, they are causative agents of serious human and animal diseases, and more importantly grow on commercial culture media [13, 30, 33]. Therefore, these factors encouraged the current evaluation of the potential effects of surfactin on the phytoplasma associated with WBDL. Thus, four surfactin-producing *B. subtilis* strains were used that had been isolated and characterized in previous work by the present authors [26]. The HPLC results showed that the strains produced more than 1,000 mg of surfactin per liter of broth medium, which was comparable with the previously reported findings [18, 26, 31].

To quantify the phytoplasma populations before and after the surfactin injections, a real-time PCR technique was used that was able to provide an estimate of the phytoplasma concentration in the plant tissues, as the standard curves confirmed the linearity of the quantification process between the exponential increases of the DNA copy numbers (from 10^1 to 10^7) and the real-time PCR threshold cycles. Consequently, the current real-time PCR results confirmed those of Torres *et al*. [29] and Kim and Wang [23], establishing this technique as a reliable criterion for the quantification of phytoplasmas in the phloem of different plants.

Finally, based on the findings of this study, tetracycline and surfactin were both found to be capable of controlling this destructive parasite of plant phloem and WBDL agent. In addition, tetracycline and surfactin were both found to have a toxic effect on the phytoplasma agent of WBDL. In previous studies [7, 8, 36], tetracycline foliar spraying, root absorption, and/or scion dipping were shown to temporarily control some other phytoplasma agents in a few plants. Yet high concentrations of tetracycline were also shown to cause phytotoxicity. However, in the present study, the application of surfactin alone and in combination with tetracycline did not cause any phytotoxicity, which may be an advantage of surfactin as an anti-phytoplasma agent. Notwithstanding, the present results could be affected by the type of plant material tested (woody or herbaceous, scions with large or narrow stem), differences in the sensitivities of the phytoplasma employed and differences in the type of experimental design and/or concentration of the antibiotics applied.

It is important to note that in the present study no phytotoxicity was observed for 150 mg/l tetracycline treatments. Moreover, the combination of surfactin and tetracycline had synergic effects, showing the highest inhibitory effect on the phytoplasma populations, and after 3 months new healthy branches appeared on the treated plants. Therefore, the simultaneous application of both metabolites could have the highest efficiency for controlling the phytoplasmal agent of WBDL. From these results, it can also be concluded that the simultaneous application of two or more metabolites and/or antibiotics could have a stronger toxicity effect on phytoplasmas.

One of the most important challenges faced when applying antibiotics in the agriculture sector is the impact on food safety. Therefore, further safety experiments related to the use of surfactin are required to address this issue. Moreover, another important problem related to using surfactin as an anti-phytoplasma agent is that the production and extraction of surfactin are very time consuming and expensive. Thus, isolating and transferring the major genes involved in the surfactin production pathway to lime plants using genetic engineering methodologies could be an efficient strategy for controlling this important disease. Further studies are also needed to understand the mechanism and mode of action/interaction of surfactin with phytoplasma cell membranes.

Acknowledgments

The authors would like to thank Dr. Mohsen Mardi, Dr. Meisam Tabatabaei, Dr. Khayam Nekoui, and Dr. Rahim Osfoori for their support, technical assistance, and critical review of the manuscript. This work was supported by a grant from the Iranian Witches' Broom Disease of Lime Program and Agricultural Research and Education Organization of Iran (AREO).

REFERENCES

1. Abdel-Mawgoud, A. M., M. M. Aboulwafa, and N. Hassouna. 2008. Optimization of surfactin production by *Bacillus subtilis* isolate BS5. *Appl. Biochem. Biotechnol.* **150:** 305–325.
2. Aldaghi, M., S. Massart, O. Dutrecq, A. Bertaccini, M. H. Jijakli, and P. Lepoivre. 2009. A simple and rapid protocol of crude DNA extraction from apple trees for PCR and real-time PCR detection of '*Candidatus* Phytoplasma mali'. *J. Virol. Methods* **156:** 96–101.
3. Alhudaib, K., Y. Arocha, M. Wilson, and P. Jones. 2009. Molecular identification, potential vectors and alternative hosts of the phytoplasma associated with a lime decline disease in Saudi Arabia. *Crop Prot*. **28:** 13–18.
4. Arocha, Y., B. Piñol, K. Acosta, R. Almeida, J. Devonshire, A. Van de Meene, E. Boa, and J. Lucas. 2009. Detection of phytoplasma and potyvirus pathogens in papaya (*Carica papaya* L) affected with 'Bunchy Top Symptom' (BTS) in eastern Cuba. *Crop Prot*. **28:** 640–646.
5. Bove, J. M., L. Danet, K. Bananej, N. Hassanzadeh, M. Taghizadeh, M. Salehi, and M. Garnier. 2000. Witches' broom disease of lime (WBDL) in Iran. *Proceeding of the 14th Conference of the International Organization of Citrus Virologists,* Riverside, California. pp. 207–212.
6. Bertaccini, A. 2007. Phytoplasmas: Diversity, taxonomy, and epidemiology. *Front. Biosci.* **12:** 673–689.
7. Chiesa, S., S. Prati, G. Assante, D. Maffi, and P. A. Bianco. 2007. Activity of synthetic and natural compounds for phytoplasma control. *Bull. Insectol.* **60:** 313–314.
8. Chung, B. N. and G. S. Choi. 2002. Elimination of aster yellows phytoplasma from *Dendranthema grandiflorum* by application of oxytetracycline as a foliar spray. *Plant Pathol. J.* **18:** 93–97.
9. Davies, D. A., H. C. Lynch, and J. Varley. 2001. The application of foaming for the recovery of surfactin from *Bacillus subtilis* ATCC 21332 cultures. *Enzyme Microb. Technol.* **28:** 346–354.
10. Desai, J. D. and I. M. Banat. 1997. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* **61:** 47–64.
11. Deng, S. J. and C. Hiruki. 1991. Genetic relatedness between two nonculturable mycoplasma-like organisms revealed by nucleic acid hybridyzation and polymerase chain reaction. *Phytopathology* **81:** 1475–1479.
12. Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19:**11–15.
13. Fleckenstein, E., C. C. Uphoff, and H. G. Drexler. 1994. Effective treatment of mycoplasma contamination in cell lines with enrofloxacin (Baytril). *Leukemia* **8**: 1424–1434.
14. Garnier, M., L. Zreik, and J. M. Bove. 1991. Witches broom, a lethal mycoplasmal disease of lime in Sultanate of Oman and the United Arab Emirates. *Plant Dis.* **75:** 546–551.
15. Ghosh, D. K., A. K. Das, S. Singh, S. J. Singh, and Y. A. Ahlawat. 1999. Occurrence of witches' broom, a new phytoplasma disease of acid lime (*Citrus aurantifolia*) in India. *Plant Dis.* **83:** 302.
16. Griffiths, H. M., W. A. Sinclair, C. D. Smart, and R. E. Davis. 1999. The phytoplasma associated with ash yellows and lilac witches'-broom: '*Candidatus* phytoplasma fraxini'. *Int. J. Syst. Bacteriol.* **49:** 1605–1614.
17. Gundersen, D. E., I. M. Lee, D. A. Schaff, N. A. Harrison, C. J. Chang, R. E. Davis, and D. T. Kingsbury. 1996. Genomic diversity and differentiation among phytoplasma strains in 16S rRNA groups I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). *Int. J. Syst. Bacteriol.* **46:** 64–75.
18. Hsieh, F. C., M. C. Li, T. C. Lin, and S. S. Kao. 2004. Rapid detection and characterization of surfactin-producing *Bacillus subtilis* and closely related species based on PCR. *Current Microbiol.* **49:** 186–191.
19. Ishiie, T., Y. Doi, K. Yora, and H. Asuyama. 1967. Suppressive effects of antibiotics of tetracycline group on symptom development of mulberry dwarf disease. *Ann. Phytopathol. Soc. Jpn.* **33:** 267–275.
20. Jones, P. 2002. Phytoplasma plant pathogens, pp. 126–139. *In* M. Waller, J. M. Lenné, and S. J. Waller (eds.). *Plant Pathologists Pocketbook*, Part 12. CAB International, Oxford University Press, USA.
21. Jung, H. Y., T. Sawayanagi, S. Kakizawa, H. Nishigawa, W. Wei, K. Oshima, *et al*. 2003. '*Candidatus* phytoplasma ziziphi', a novel phytoplasma taxon associated with jujube witches'-broom disease. *Int. J. Syst. Evol. Microbiol.* **53:** 1037–1041.
22. Kim, S., J. Y. Kim, S. H. Kim, H. J. Bae, H. Yi, S. H. Yoon, *et al*. 2007. Surfactin from *Bacillus subtilis* displays anti-proliferative effect *via* apoptosis induction, cell cycle arrest and survival signalling suppression. *FEBS Lett.* **581:** 865–871.
23. Kim, J. and N. Wang. 2009. Characterization of copy numbers of 16S rDNA and 16S rRNA of *Candidatus Liberibacter asiaticus* and the implication in detection in planta using quantitative PCR. *BMC* **2:** 37.
24. Lim, J. H., B. K. Park, M. S. Kim, M. H. Hwang, M. H. Rhee, S. C. Park, and H. I. Yun. 2005. The anti-thrombotic activity of surfactins. *J. Vet. Sci.* **6:** 353–355.
25. Mireles, J. R., A. Toguchi, and R. M. Harshey. 2001. *Salmonella enterica* serovar Typhimurium swarming mutants with altered biofilm-forming abilities: Surfactin inhibits biofilm formation. *J. Bacteriol.* **183:** 5848–5854.
26. Mohamadipour, M., M. Mousivand, Gh. Salehi Jouzani, and S. Abasalizadeh. 2009. Molecular and biochemical characterization of Iranian surfactin producing *Bacillus subtilis* isolates and evaluation of their biocontrol potential against *Aspergillus flavus* and *Colletotrichum gleosporiodes. Can. J. Microbiol.* **55:** 395–404.
27. Navrátil, M., D. Safárová, P. Válová, J. Fránová, and M. Simková. 2009. Phytoplasma associated with witches'-broom disease of *Ulmus minor* MILL in the Czech Republic: Electron microscopy and molecular characterization. *Folia Microbiol. (Praha)* **54:** 37–42.
28. Pagadoy, M., F. Peypoux, and J. Wallach. 2005. Solid-phase synthesis of surfactin, a powerful biosurfactant produced by *Bacillus subtilis* and of four analogues. *Int. J. Peptide Res. Therap.* **11:** 195–202.
29. Torres, E., E. Bertolini, M. Cambra, C. Monton, and M. P. Martin. 2005. Real-time PCR for simultaneous and quantitative detection of quarantine phytoplasmas from apple proliferation (16SrX) group. *Mol. Cell. Probes* **19:** 334–340.

30. Vollenbroich, D., G. Pauli, M. Ozel, and J. Vater. 1997. Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis. Appl. Environ. Microbiol*. **63:** 44–49.
31. Wei, Y., C. Lai, and J. Chang. 2007. Using Taguchi experimental design methods to optimize trace element composition for enhanced surfactin production by *Bacillus subtilis* ATCC 21332. *Process Biochem.* **42:** 40–45.
32. Whang, L. M., P. W. G. Liu, C. C. Ma, and S. S. Cheng. 2008. Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. *J. Hazard. Mater*. **151:** 155–163.
33. Xu, Q., G. Tian, Z. Wang, F. Kong, Y. Li, and H. Wang. 2009. Molecular detection and variability of jujube witches'-broom phytoplasmas from different cultivars in various regions of China. *Wei Sheng Wu Xue Bao* **49:**1510–1519.
34. Zhao, Y., Q. Sun, W. Wei, R. E. Davis, W. Wu, and Q. Liu. 2009. *Candidatus* phytoplasma tamaricis', a novel taxon discovered in witches'-broom-diseased salt cedar (*Tamarix chinensis* Lour). *Int. J. Syst. Evol. Microbiol.* **59:** 2496–2504.
35. Zreik, L., P. Carle, J. M. Bové, and M. Garnier. 1995. Characterization of the mycoplasma-like organism associated with witches'-broom disease of lime and proposition of a *Candidatus* taxon for the organism, "*Candidatus* phytoplasma aurantifolia". *Int. J. Syst. Bacteriol.* **45:** 449–453.
36. Zaim, M. and A. Samad. 1995. Association of phytoplasmas with a witches-broom disease of *Withania somnifera* (L) Dunal in India. *Plant Sci.* **109:** 225–229.