Rhamnolipid produced by *Pseudomonas aeruginosa* SS14 causes complete suppression of wilt by *Fusarium oxysporum* f. sp. *pisi* in *Pisum sativum*

Siddhartha Narayan Borah · Debahuti Goswami · Jiumoni Lahkar · Hridip Kumar Sarma · Mojibur Rahman Khan · Suresh Deka

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**Abstract**  *Fusarium oxysporum* f. sp. *pisi* (van Hall) Snyder & Hansen is an important pathogen of pea that causes wilt. The present study was carried out to evaluate the efficacy of rhamnolipid biosurfactant produced by newly isolated *Pseudomonas aeruginosa* strain SS14 as an antifungal agent against *F. oxysporum* f. sp. *pisi* in *Pisum sativum* L. The bacterial strain *P. aeruginosa* SS14 was isolated from crude oil contaminated soil and identified by 16S rDNA sequencing. The biosurfactant was characterized as rhamnolipid by FTIR and LC–MS analyses. Treatment of pea seeds and seedlings under natural conditions of light, temperature and humidity with the rhamnolipid at a concentration of 25 μg ml⁻¹ prior to sowing or planting in pathogen laden soil resulted in complete suppression of characteristic wilt symptoms. The results demonstrate the possibility to develop a sustainable and eco-friendly control measure against *F. oxysporum* f. sp. *pisi* which is currently not available.

**Keywords**  *Fusarium oxysporum* f. sp. *pisi* · *Pseudomonas aeruginosa* strain SS14 · *Pisum sativum* · Rhamnolipid · Antifungal agent

**Introduction**

*Fusarium oxysporum* in its various pathogenic forms (*formae speciales*) has been described to be the most damaging species of the genus from the standpoint of plant diseases. This species also happens to be the predominant inhabitant of cultivated fields within the genus (Smith 2007). *Fusarium oxysporum* f. sp. *pisi* (van Hall) Snyder & Hansen (*Fop*) is an important pathogen of pea (*Pisum sativum* L.), causing wilt of plants often resulting in partial to complete loss of crop (Sharma 2011). The severity of the disease is often elevated when short rotation of pea with other crops is practiced, because this soil-borne pathogen can survive as thick-walled chlamydospores in soil with viability of more than ten years (Kraft 1994). This allows the pathogen to build up a sufficient inoculum in the soil which causes severe crop losses upon subsequent plantation of a susceptible cultivar. Hence the prevention of Fusarium wilt has occupied a thrust area in the agricultural research.
Fusarium wilt being a soil-borne disease cannot be effectively controlled using fungicides (Sharma et al. 2010). Consequently, the control measures against Fop are mostly reliant on the use of resistant pea cultivars, and many researchers therefore have concentrated on the development and identification of resistant cultivars (Sharma et al. 2010; Bani et al. 2012). Although the use of these resistant pea cultivars has proven to be an effective approach in controlling this disease, yet there is a constant risk of breakdown of resistance because of the emergence of new variants of pathogen (Bani et al. 2012). This associated risk underlines the necessity to explore other control measures to overcome the menace. It is noteworthy that potential biocontrol measures against other formae speciales of F. oxysporum have been reported involving the use of non-pathogenic or protective strains of F. oxysporum (Alabouvette et al. 2009), which however have not been reported against Fop. Another well-characterized group of microorganisms widely used for the purpose of biocontrol are the fluorescent pseudomonads. The antifungal properties of pseudomonads against Fusarium species have been reported earlier by other researchers (Bradley and Punja 2010). Pseudomonads are indigenous in the environment and produce secondary metabolites that are inhibitory to plant pathogens (Haas and Defago 2005). Important groups of secondary metabolites produced by fluorescent pseudomonads include antibiotics and biosurfactants.

Biosurfactants are surface active microbial metabolites classified broadly into glycolipids, lipopeptides, neutral lipids, polymeric biosurfactants, fatty acids and phospholipids based on their molecular structure (Cameotra et al. 2010). Biosurfactants represent ecological alternatives with distinct advantages over their synthetic counterparts, such as biodegradability, possible production from renewable resources, lower or non-toxicity, high specificity and stability over a wide range of temperature (−18 to 121 °C), pH (2–12) and salinity (NaCl concentrations up to 20 %) (Nitschke and Costa 2007). One of the most widely studied biosurfactant over the years has been the rhamnolipids (Abdel-Mawgoud et al. 2010). Rhamnolipids have been reported to exhibit antimicrobial and antifungal properties (Abalos et al. 2001). Goswami et al. (2014) reported the use of rhamnolipid biosurfactant against Fusarium sacchari. However, the use of rhamnolipid as antifungal agent against Fop has not been reported earlier. Therefore, the present study was carried out with the aim to assess the efficacy of rhamnolipid as prospective antifungal agent against Fop.

Materials and methods

Fungal strain

Fusarium oxysporum f. sp. pisi (van Hall) Snyder & Hansen strain 4814 was procured from ITCC (Indian type culture collection), Indian agricultural research Institute (IARI), New Delhi, India. The fungal strain was stored as microconidial suspensions at −80 °C in 30 % glycerol. The working cultures were maintained in Potato dextrose broth (PDB; HiMedia) and Potato dextrose agar (PDA; HiMedia) plates at 4 °C and were subcultured every two weeks.

Bacterial strain

The bacterial strain Pseudomonas aeruginosa strain SS14 (GenBank accession no. KC866140, NCBI) was isolated from crude oil contaminated soil of Lakowa in Sivasagar district of Assam, India (27°04′44″N, 94°51′17″E; altitude 95 m) following the methodology of Goswami et al. (2014). The strain was chosen from a total of eight biosurfactant producing isolates that were screened on the basis of highest surface tension reduction and inhibition of mycelial growth of Fop.

Biosurfactant production

The cell free culture supernatant of P. aeruginosa strain SS14 was used for screening of biosurfactant production through different methods like oil displacement test, drop collapsing test and emulsification assay based on the methodology previously described by Sriram et al. (2011) with slight modifications: crude oil was used for drop collapsing test, while emulsification assay was carried out against crude oil and olive oil. Production of biosurfactant was further confirmed by measuring the reduction of surface tension of the culture medium from 70 to 35 mN m⁻¹ or less using K11 tensiometer (Kruss, Hamburg, Germany). All the experiments were performed in triplicates and repeated once.
Production medium and cultivation conditions

The bacterial inoculum was prepared in nutrient broth (NB) medium (HiMedia, Mumbai, India) and incubated at 35 °C for 24 h. A 5 ml inoculum from the seed culture was used to inoculate the biosurfactant production medium of 100 ml in a 500 ml Erlenmeyer flask and was incubated at 150 rpm at 35 °C for two days. The production medium used in the experiment comprised of the following (g l⁻¹): NaNO₃ (4.0), KCl (0.1), KH₂PO₄ (0.5), K₂HPO₄ (1.0), CaCl₂ (0.01), MgSO₄·7 H₂O (0.5), FeSO₄·7 H₂O (0.01), Yeast extract (0.1) and 10 ml of trace element solution containing (g l⁻¹): H₃BO₃ (0.26), CuSO₄·5 H₂O (0.5), MnSO₄·H₂O (0.5), (NH₄)₆Mo₇O₂₄·4 H₂O (0.06) and ZnSO₄·7 H₂O (0.7). The pH of the medium was adjusted to 7.0 ± 0.2. Glucose at 2 % (w/v) was added as sole carbon source to the production medium.

Extraction of biosurfactant

Biosurfactant produced by the bacteria was extracted by following a modified method described earlier by George and Jayachandran (2008). Bacterial cells were removed from the culture medium by centrifugation at 10,000 rpm (15,120 g) for 20 min at 4 °C. Cell-free culture broth was then deproteinized by heating at 110 °C for 10 min. After cooling, the broth was acidified to pH 3.0 by the addition of 2 N HCl. Biosurfactants were extracted continuously from the acidified cell-free culture broth using ethyl acetate (1:1) at room temperature. The mixture was shaken vigorously and left static for 10 min for phase separation. The organic phase was then transferred to a rotary evaporator (Equiton-Roteva, Mumbai, India) and a viscous honey-coloured product was recovered after solvent evaporation at 40 °C under reduced pressure.

Selection of suitable carbon source

In the present study, the most suitable carbon source for the production medium was selected based on the in vitro antifungal activity of the extracted biosurfactant against mycelial growth of *Fop*. Six different carbon sources namely glucose, mannitol, glycerol, sucrose, molasses and n-hexadecane were used in this experiment to screen variations. The crude biosurfactant was extracted from 100 ml production medium for each carbon source and was dissolved in 100 ml Milli-Q water (pH 7) at a concentration equivalent to that of the carbon source with the lowest production. This was done to maintain equal concentration of crude biosurfactant throughout the range of treatments for each carbon source. Then 3.9 g PDA was added to the medium and sterilized. Sterilized medium was poured onto 90 mm petri dishes. Control plates were prepared with PDA in Milli-Q water. A 6 mm plug of *Fop* from ten days old culture was transferred onto the middle of the plates. The plates were then incubated at 25 °C. Diameter of mycelial growth was measured after ten days of inoculation when the control plates were completely covered by mycelia and were compared to the plates under observation to determine percentage of inhibition.

Purification of biosurfactant

The crude extract obtained in production medium with mannitol as sole carbon source was purified by liquid column chromatography in a 26 × 3.3 cm² column containing 50 g of activated silica gel 60-chloroform slurry. The column was loaded with 1 g sample of crude biosurfactant prepared in 5 ml CHCl₃ and washed with chloroform until the neutral lipids were completely eluted. This was followed by chloroform:methanol mobile phases separation in sequence: 50:3 v/v (500 ml), 50:5 v/v (300 ml), and 50:50 v/v (300 ml) maintained at a flow rate of 1 ml min⁻¹. Finally, 20 ml fractions were collected and tested individually for presence of biosurfactant through measurement of surface tension. A final wash with 50:50 chloroform:methanol removed any traces of remaining biosurfactant from the column. Biosurfactant containing fractions were combined and dried under vacuum in a rotary evaporator at 40 °C under reduced pressure.

Characterization of biosurfactant

Primary characterization of the biosurfactant was carried out using Molisch’s and Emulsion tests following the methodology of Goswami et al. (2014). Further characterization was accomplished with the help of FTIR and LC–MS analyses.

Fourier transform infrared spectroscopy (FTIR) was performed to determine the functional groups and the type of bonds present in the biosurfactant. A 5 mg
of column purified biosurfactant was ground to fine powder with 100 mg of spectral-grade KBr (Merck, Germany) and pressed with 6,000 kg cm\(^{-2}\) pressure for 120 s to obtain a translucent KBr pellet. The sample pellet was subjected to FTIR analysis in a Nicolet 6700 FT-IR System, USA. The spectra of the sample were collected at a resolution and wave number accuracy of 4 and 0.01 cm\(^{-1}\), respectively, and 32 scans with correlation for atmospheric CO\(_2\).

The biosurfactant mixtures were separated and different structural analogues were identified by liquid chromatography–mass spectrometry (LC–MS, Agilent Technologies 1260 Infinity LC and 6410 Triple Quad MS). The column purified biosurfactant sample was dissolved in methanol and 2 µl aliquot was injected into a ZORBAX C18 column (2.1 × 50 mm\(^2\)). The LC flow rate was maintained at 0.2 ml min\(^{-1}\). An acetonitrile/water gradient with 0.01 % formic acid was used (10–90 %) as the mobile phase. ESI–MS was performed in positive ion mode and analyzed using Agilent software. Full scan data were obtained by scanning from m/z 100–950 with a fragmentor voltage calibrated at 135.0 V.

Commercially available rhamnolipid R–95 (Sigma-Aldrich, USA) was also analyzed by LC–MS following the same methodology. This was done to compare the differences in the composition of rhamnolipidic structural analogues between the commercial rhamnolipid and the sample under study and the subsequent effects brought about in their activity against *Fop*.

**Microtiter assay to evaluate in vitro inhibition activity against *Fop***

This experiment was conducted to compare the antifungal activities of crude biosurfactant (BS) and column purified biosurfactant (PBS). Rhamnolipid R-95 was used as reference standard. A 20 µl spore suspension of *Fop* \((10^7 \text{ cfu ml}^{-1})\) prepared in PDB was used to inoculate 96-well microtiter plates containing 100 µl of BS, PBS and R-95 at concentrations of 5, 10, 25, 50 and 100 µg ml\(^{-1}\) in PDB. The tests were performed with positive control (only PDB), negative control (PDB + spore suspension) and sterility control (PDB + BS/PBS/R-95). Plates were incubated at 25 °C for 48 h. Fungal growth was evaluated by measuring OD\(_{600 \text{ nm}}\) in a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA) and the percentage inhibition was determined for all three treatments.

**Plant bioassays**

The commercially available cultivar Azad P-1 was selected for the present study. Pea seeds were surface-sterilized for 20 min in a 2 % solution of sodium hypochlorite and then rinsed three times with sterile water. The seeds were wrapped in wet filter paper in a Petri dish, stratified for two days at 4 °C in the dark and incubated at 26 ± 2 °C until germination (Bani et al. 2012). Once germinated, the seedlings were transferred to pots of size 20 × 15 cm (diameter × height) containing 2 kg sterile soil and were grown under natural conditions of light, temperature and humidity. The experiments were conducted in the months of November–December when temperature and humidity ranged between 18 and 24 °C and 75–80 % respectively.

The pot assay for evaluation of antifungal activity of the crude biosurfactant (BS) against *Fop* was setup in plastic pots of size 8 × 10 cm (diameter × height). Each pot was filled with 250 g sterile soil. The soil was artificially infested with the test pathogen by inoculating with 100 ml spore suspension of \(10^7\) cfu ml\(^{-1}\) prepared in PDB. Control pots were inoculated with sterile distilled water and uninoculated PDB. *In planta* antifungal effect of the biosurfactant was evaluated by two different tests, one by root treatment of seedlings and another by seed treatment with BS before introduction of the respective seedlings and seeds into infected soil. Each treatment set consisted of four replicate pots with one plant in each pot and the experiment was repeated once.

**Root treatment with crude biosurfactant**

For this experiment, seven days-old pea seedlings were used. The seedlings were carefully uprooted to prevent breaking of the stems. The excess soil from the roots was removed by washing several times in sterilized distilled water. Thereafter, the roots were dipped in BS (crude extract obtained in production medium with mannitol) solutions of five different concentrations of 5, 10, 25, 50 and 100 µg ml\(^{-1}\) (w/v in dH\(_2\)O) for 1 h. Control seedlings were dipped in sterilized water for the same duration. Then the seedlings were planted in *Fop* infected soil in the experimental pots and kept under natural conditions as
described earlier. Observations were taken for the next ten days post-planting (dpp). Seedlings planted in soil inoculated with sterilized dH2O and PDB served as positive control. Assessment for characteristic symptoms was done in terms of disease incidence and dry biomass of the plants. Disease incidence (percentage of diseased leaves) was calculated by counting the number of infected/wilted leaf to the total number of leaves present in the plant. The results were plotted in terms of percentage of disease inhibition. For measuring the dry biomass, plants were carefully removed from the pot keeping the roots intact; pulling was avoided to prevent damaged roots from breaking off. Plants were cleaned with tap water to remove excess soil and were dried in the shade for seven days. The weight of the dried plants were measured and tabulated.

Seed treatment with crude biosurfactant

Surface sterilized seeds were soaked overnight in BS solutions of the same concentrations as those used for the root treatment. Control seeds were soaked in sterilized water. The seeds were subsequently sown in Fop infected soil and kept under similar natural conditions. Assessment was made on the basis of seed germination, fruiting and dry biomass compared to those of the control treatments sown in pathogen laden and pathogen free soils.

Statistical analysis

The effect of BS concentration on disease inhibition and biomass of seedlings was analysed using one-way ANOVA with post-hoc pairwise Least Significant Difference (LSD) comparison at significant level of 0.05 using the Statistical Package for the Social Sciences (SPSS 18.0, SPSS Inc, Chicago, USA).

Results

Production of biosurfactant and selection of suitable carbon source

To confirm that P. aeruginosa SS14 was producing a biosurfactant, it was grown in a production medium with glucose as sole carbon source and the surface tension of the culture was measured over a span of 120 h at 24 h intervals. The surface tension decreased from an initial value of 70–28.1 mN m⁻¹ after 48 h of incubation. This finding confirmed the ability of the bacterial strain to produce biosurfactants (Banat 1995). Biosurfactant production could be confirmed further by positive results obtained from oil displacement test (3.75 ± 0.19 cm²), drop collapsing test and emulsification assay (100 ± 0.0 % and 57.33 ± 0.57 % against crude oil and olive oil respectively). The amount of crude biosurfactant extracted from the media containing carbon sources glucose, mannitol, glycerol, sucrose, n-hexadecane and molasses was found to be 2.33, 2.29, 2.30, 0.66, 0.88 and 0.86 g l⁻¹ respectively. Accordingly, the crude biosurfactant was used at a concentration of 0.66 g l⁻¹ for each carbon source during the evaluation of antifungal activity against Fop for selection of the most suitable carbon source. Mannitol was found to be the most suitable carbon source evaluated on the basis of the highest percentage of inhibition (96.68 %) of mycelial growth of Fop. Percentage of inhibition was the lowest in case of n-hexadecane (11.11 %). The results are presented in Fig. 1.

Characterization of biosurfactant

The initial characterization of the biosurfactant as obtained by Molisch’s and Emulsion tests revealed a
glycolipidic composition. The presence of carbohydrate components in the biosurfactant was confirmed by the appearance of a clear purple ring between the layers of solvent in the Molisch’s test, while a cloudy white emulsion during emulsion test confirmed the presence of lipids in the sample.

FTIR spectroscopy revealed important band spectra at 3,366, 2,925, 1,732 and 1,037 cm\(^{-1}\) (Fig. 2a) confirming the chemical structure of the biosurfactant to be identical to those of other reported rhamnolipids, which comprises of rhamnose rings and long hydrocarbon chains (Pornsunthornthawee et al. 2008). The spectral band at 3,366 cm\(^{-1}\) corresponds to –OH stretching of the hydroxyl group. The strong absorption peak at 2,925 cm\(^{-1}\) indicated the presence of –CH aliphatic stretching bands. The presence of ester compounds was confirmed by the characteristic peak observed at 1,732 cm\(^{-1}\) corresponding to –C=O vibrations of carbonyl groups and –OH stretching of hydroxyl group, –CH peaks at 3,366.20, 2,925.52, 1,732.14 and 1,037.86 cm\(^{-1}\). Observation of –C–O– stretching band at 1,037 cm\(^{-1}\) confirmed the presence of lipids in the sample. FT-IR spectra of the biosurfactant revealing important bands at 3,366, 2,925, 1,732 and 1,037 cm\(^{-1}\) (Fig. 2) confirmed the presence of lipids in the sample.

Fig. 2 FT-IR spectra of the biosurfactant revealing important peaks at 3,366.20, 2,925.52, 1,732.14 and 1,037.86 cm\(^{-1}\) corresponding to –OH stretching of hydroxyl group, –CH aliphatic stretching, –C=O vibrations of carbonyl groups and –C–O– vibrations respectively.

(Abdel-Mawgoud et al. 2010; Pantazaki et al. 2011; Pereira et al. 2012). The LC–MS spectra of the purified rhamnolipid revealed the predominance of mono-rhamnolipids with the distribution of intense protonated ions [M + H]\(^+\) at m/z 317, 335, 357, 449 and 503 corresponding to Rha-C\(_9\)-C\(_2\), Rha-C\(_10\), Rha-C\(_12\)-3, Rha-C\(_8\)-C\(_8\) and Rha-C\(_10\)-C\(_10\)/Rha-C\(_10\)-C\(_10\) respectively. The di-sodium adduct ion [M – H + 2Na]\(^+\) at m/z 521 corresponds to Rha-C\(_10\)-C\(_8\)/Rha-C\(_8\)-C\(_10\). The ion at m/z 679 corresponds to di-rhamnolipid Rha\(_2\)-C\(_10\)-C\(_10\)/Rha\(_2\)-C\(_12\)-C\(_10\), while a sodiated fatty acyl adduct, i.e. [M + Na]\(^+\) formed by the cleavage of the C\(_12\) fatty acid chain from the di-rhamnolipidic structural analogue, was detected at m/z 239. These results clearly indicated that the biosurfactant produced by the strain SS14 is a mixture of mono and di-rhamnolipidic structural analogues. The LC–MS spectra of the commercial rhamnolipid R-95 revealed a total of 15 rhamnolipidic structural analogues, 11 mono and four di-rhamnolipids. The spectra predominantly exhibited [M + H]\(^+\) pseudo molecular ions, accompanied by the adduct ions [M + Na]\(^+\), [M + K]\(^+\), [M – H + 2Na]\(^+\), [M + H + Na]\(^+\) and [M + H + 2Na]\(^+\). [M + H]\(^+\) ions at m/z 319, 335, 359, 387,473 and 645 correspond to mono–rhamnolipids Rha-C\(_9\)-C\(_1\), Rha-C\(_10\), Rha-C\(_12\)-3, Rha-C\(_14\)-2, Rha-C\(_8\)-C\(_10\)/Rha-C\(_10\)-C\(_8\) and Rha-C\(_14\)-C\(_10\)/Rha-C\(_10\)-C\(_14\), respectively. The [M + H]\(^+\) ion at m/z 479 corresponds to di-rhamnolipid Rha\(_2\)-C\(_10\)-C\(_10\). The ions at m/z 499 and 521 correspond to mono–rhamnolipids Rha-C\(_9\)-C\(_1\), Rha-C\(_10\), Rha-C\(_12\)-3, Rha-C\(_14\)-2, Rha-C\(_8\)-C\(_10\)/Rha-C\(_10\)-C\(_8\) and Rha-C\(_14\)-C\(_10\)/Rha-C\(_10\)-C\(_14\), respectively. The [M + K]\(^+\) ions at m/z 341 and 661 correspond to potassium adduct ions of mono and di-rhamnolipids Rha-C\(_9\)-C\(_2\) and Rha\(_2\)-C\(_10\)-C\(_8\)/Rha\(_2\)-C\(_8\)-C\(_10\), respectively. The [M – H + 2Na]\(^+\) di-sodium ion at m/z 575 corresponds to Rha-C\(_10\)-C\(_12\)-C\(_10\). The ions at m/z 527 and 543 correspond to sodiated and potassiated adduct ions of the same mono-rhamnolipid analogue Rha-C\(_10\)-C\(_10\). The ions at m/z 499 and 521 correspond to sodiated and di-sodiarted ions of the same mono-rhamnolipid analogue Rha-C\(_8\)-C\(_10\)/Rha-C\(_10\)-C\(_8\). Rha-C\(_10\)-C\(_14\)-2 and Rha-C\(_8\)-C\(_10\)-C\(_14\)-2, respectively. There were three structural analogues present both in the test as well as commercial sample, i.e. Rha-C\(_10\) (m/z 335), Rha-C\(_8\)-C\(_10\)/Rha-C\(_10\)-C\(_8\) (m/z 521) and Rha\(_2\)-C\(_10\)-C\(_12\)/Rha\(_2\)-C\(_12\)-C\(_10\) which was detected as [M + H]\(^+\) (m/z 679) in the test and [M + Na]\(^+\) (m/z 701) in the commercial sample. 

[Image: 50x130 to 259x262]
Microtiter assay to evaluate in vitro inhibition activity against Fop

The results of the microtiter assay are presented in Fig. 3. The results revealed that the antifungal activity of BS and PBS were comparable throughout the range of concentrations with no significant difference when evaluated statistically ($F_{1,28} = 0.021$, $P > 0.05$). However, these results were significantly better than that of R-95 rhamnolipid standard ($F_{2,42} = 5.106$, $P < 0.05$).

Antifungal activity of crude biosurfactant against Fop in plant bioassays

Root treatment

The assessment of disease symptoms of the untreated seedlings planted in pathogen laden soil (negative control) depicted that the initial symptoms on the primary leaves appeared around 3–5 dpp and sequentially reached the younger leaves until the whole plant withered and died 10 dpp. This was also characterized by reduced growth rate and subsequent reduction of biomass ($F_{7,24} = 2363.62$, $P < 0.05$). The diseased plants also exhibited a severely destructed root system in comparison to those of healthy plants. The seedlings treated with BS concentrations of 100, 50 and 25 µg ml$^{-1}$ exhibited completely healthy growth for the entire duration of the experiment characterized by healthy leaves and increased biomass (100 % disease inhibition) comparable to those of the control seedlings planted in uninfected soil ($F_{4,15} = 0.663$, $P > 0.05$). The seedlings treated with 10 µg ml$^{-1}$ BS developed characteristic symptoms on the lower leaves at 8 dpp but no advancement of the symptoms could be observed later, accounting for 80 % disease inhibition by the end of the experiment. There was no inhibitory effect of the BS on Fop at the concentration of 5 µg ml$^{-1}$ (0 % disease inhibition) as the seedlings developed characteristic symptoms around 4–5 dpp and died rapidly. The results are presented in Table 1.

Seed treatment

Untreated seeds sown in the pathogen free soil (positive control) germinated three–four days post-sowing (dps) while those sown in the pathogen laden soil (negative control) completely failed to germinate. Initiation of fruiting was observed at around 34–35 dps for the control plants. Seeds treated with BS concentrations of 25, 50 and 100 µg ml$^{-1}$ exhibited comparable ($F_{4,15} = 1.050$, $P > 0.05$) observations to those of positive control plants. There was no significant delay in germination of the seeds treated with 10 µg ml$^{-1}$ BS when compared to control seeds ($F_{2,9} = 0.9$, $P > 0.05$), but the plants developed characteristic disease symptoms in the leaves at

<table>
<thead>
<tr>
<th>Sample</th>
<th>Disease inhibition (%)</th>
<th>Dry weight (in g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0 ± 0.0 a</td>
<td>0.0799 ± 0.0011  c</td>
</tr>
<tr>
<td>Positive control (dH$_2$O)</td>
<td>100 ± 0.0 b</td>
<td>0.1582 ± 0.0017  a</td>
</tr>
<tr>
<td>Positive control (PDB)</td>
<td>100 ± 0.0 b</td>
<td>0.1565 ± 0.0010  a</td>
</tr>
<tr>
<td>100 µg ml$^{-1}$</td>
<td>100 ± 0.0 b</td>
<td>0.1573 ± 0.0012  a</td>
</tr>
<tr>
<td>50 µg ml$^{-1}$</td>
<td>100 ± 0.0 b</td>
<td>0.1576 ± 0.0012  a</td>
</tr>
<tr>
<td>25 µg ml$^{-1}$</td>
<td>100 ± 0.0 b</td>
<td>0.1576 ± 0.0013  a</td>
</tr>
<tr>
<td>10 µg ml$^{-1}$</td>
<td>80 ± 2.25 c</td>
<td>0.1422 ± 0.0007  b</td>
</tr>
<tr>
<td>5 µg ml$^{-1}$</td>
<td>0 ± 0.0 a</td>
<td>0.0845 ± 0.0013  c</td>
</tr>
</tbody>
</table>

Data are means of four replicates ± SD. Different letters within each column indicate significant difference between values according to LSD at $a = 0.05$.
around 19–20 dps and the growth appeared stunted: the plants survived as there was no visible progress of the symptoms but no fruiting was observed until termination of the experiment at 40 dps. The seeds treated with 5 μl gm l⁻¹ BS failed to germinate in three out of four replicates. Delayed germination at 7 dps was observed in the fourth replicate which developed characteristic symptoms and died within two days, i.e. 9 dps. Statistical analyses were performed for time of germination, percentage of germination, time of fruiting and dry biomass among the treatment groups in comparison to both positive and negative controls. Results of the experiment are presented in Table 2.

Discussion

In this study, suppression of Fusarium wilt in *P. sativum* cultivar Azad P-1 was studied using rhamnolipid biosurfactant produced by *P. aeruginosa* strain SS14 in terms of root and seed treatment. Both methods proved to be highly effective in suppressing the disease symptoms characteristic of *Fop* which may prove to be useful for field applications.

The biosurfactant produced by the strain SS14 was characterized to be rhamnolipid by FTIR and LC–MS analyses. The LC–MS spectra revealed seven constituent rhamnolipidic structural analogues, six predominant mono-rhamnolipids and one di-rhamnolipid. This is consistent with earlier reports wherein *P. aeruginosa* strains have been shown to produce a mixture of mono and di-rhamnolipids with varying degree of abundance of one over the other (Deziel et al. 1999; Sandoval et al. 2001). When compared with the commercial R-95 rhamnolipid, three structural analogues were found to be present both in the test as well as commercial sample, i.e. Rha-C₁₀, Rha-C₈-C₁₀/Rha-C₁₀-C₈ and Rha₂-C₁₀-C₁₂/Rha₂-C₁₂-C₁₀ among a total of seven and 15 analogues respectively. The rhamnolipid composition and predominance of a particular type of structural analogue have been observed to be dependent on the type of carbon substrate utilized (Rahman et al. 2002; Benincasa and Accorsini 2008), age of the culture and most importantly the strain of *P. aeruginosa* (Haba et al. 2003). Keeping consistency with these reports, the present analyses for two rhamnolipidic samples with different sources of origin revealed different structural analogues. The rhamnolipid produced by strain SS14 exhibited significantly better antifungal activity against *Fop* as compared to that of R-95. This difference in activity highlights the pivotal role of constituent analogues in the activity of a rhamnolipidic sample. The unique analogues present in the rhamnolipid produced by SS14 seemed to enhance the antifungal activity against *Fop* in comparison to that of R-95. Similarly, the higher antifungal activity observed while using mannitol as the sole carbon

<table>
<thead>
<tr>
<th>Sample</th>
<th>Germination of seeds</th>
<th>Time of fruiting (dps)</th>
<th>Dry weight (in g)</th>
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<tbody>
<tr>
<td></td>
<td>Time (dps)</td>
<td>Percentage (%)</td>
<td></td>
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<tr>
<td>Negative control</td>
<td>NG</td>
<td>NA</td>
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</tr>
<tr>
<td>Positive control (dH₂O)</td>
<td>3.25 ± 0.5</td>
<td>100 ± 0.0 a</td>
<td>34.0 ± 0.0</td>
</tr>
<tr>
<td>Positive control (PDB)</td>
<td>3.5 ± 0.57</td>
<td>100 ± 0.0 a</td>
<td>34.25 ± 0.5</td>
</tr>
<tr>
<td>100 μg ml⁻¹</td>
<td>3.25 ± 0.5</td>
<td>100 ± 0.0 a</td>
<td>34. 0 ± 0.0</td>
</tr>
<tr>
<td>50 μg ml⁻¹</td>
<td>3.25 ± 0.5</td>
<td>100 ± 0.0 a</td>
<td>34.5 ± 0.57</td>
</tr>
<tr>
<td>25 μg ml⁻¹</td>
<td>3.5 ± 0.57</td>
<td>100 ± 0.0 a</td>
<td>34.25 ± 0.5</td>
</tr>
<tr>
<td>10 μg ml⁻¹</td>
<td>3.75 ± 0.5</td>
<td>100 ± 0.0 a</td>
<td>NF</td>
</tr>
<tr>
<td>5 μg ml⁻¹</td>
<td>7.0 ± 0.0</td>
<td>25 ± 0.0 b</td>
<td>NF</td>
</tr>
</tbody>
</table>

Data are means of four replicates ± SD. Different letters within each column indicate significant difference between values according to LSD at α = 0.05. ANOVA was performed for time of germination (F₁₀ , ₂₁ = 0.868, P = 0.534), percentage of germination (F₁₀ , ₂₁ = 225.0, P < 0.000), time of fruiting (F₅,₁₅ = 1.050, P = 0.415) and dry weight (F₆ , ₂₁ = 3937.312, P < 0.000) NG no germination; NA not applicable; NF no fruiting; dps days post-sowing
source may be attributed to the underlying difference in constituent structural analogues of rhamnolipid for each carbon source used. The present study also revealed the more crucial role of structural analogues over that of total amount in influencing the activity of a rhamnolipid sample. The production was found to be the highest using glucose and the lowest using sucrose as sole carbon sources. However, the antifungal activity was found to be the best using mannitol which was selected as the most suitable carbon source for further studies. Rhamnolipids have been previously reported to have antifungal activity against phytopathogens including Botrytis sp., Rhizoctonia solani, Colletotrichum sp., Phytophthora sp., Fusarium sp., Phytophthora sp. and Plasmopara sp. (Stanghellini and Miller 1997; Kim et al. 2000; Varnier et al. 2009; Goswami et al. 2014). The mode of action of rhamnolipids against zoospore-producing plant pathogens is the lysis of zoospores because of intercalation with the zoospore plasma membranes which are not protected by a cell wall (De Jonghe et al. 2005). Studies by Perneel et al. (2008) and Goswami et al. (2014) have reported that rhamnolipids could effectively reduce the mycelial growth of Pythium myriotylum and F. sacchari demonstrating the fact that rhamnolipids can also have an adverse effect on cell structures that are indeed protected by a cell wall.

The external symptoms characteristic of Fusarium wilt disease includes vein clearing, leaf epinasty, wilting, chlorosis, necrosis, and abscission leading to complete plant wilting and death (MacHardy and Beckman 1983). Keeping consistency with this, the disease incidence in the present study was evaluated in terms of symptomatic leaf infections. Additionally dry biomass of the plants was also evaluated to monitor the comparability of growth to healthy plants, as stunted growth has also been described to be a prominent symptom of Fusarium wilt (Sharma 2011). The antagonistic effect of rhamnolipid against Fop was clearly illustrated by the findings of this study, as a concentration as low as 25 µg ml⁻¹ of crude rhamnolipid caused complete disease inhibition. This concentration is quite low compared to those reported in other in vivo studies involving the antifungal effect of rhamnolipid (Kim et al. 2000; De Jonghe et al. 2005). Kim et al. (2000) reported the use of pure rhamnolipid B (silica gel column purified) isolated from P. aeruginosa strain B5 against F. oxysporum f. sp. cucumber in microtiter assay which required a concentration >50 µg ml⁻¹ to cause partial suppression of the pathogen. The study also reported that a significantly higher concentration of 0.5 g l⁻¹ was required in vivo to control Phytophthora blight in pepper-growing fields. However, the development of typical lesions on leaves was observed using the same concentration on Colletotrichum orbiculare on cucumber plant. With reports of varying results for the same rhamnolipid B applications on suppression of fungal infestations, it may be inferred that antifungal activity and the lowest effective concentration of a particular biosurfactant vary with the type of pathogen under study.

Rhamnolipid or biosurfactants as a whole are highly biodegradable under natural environmental conditions. In this context, a previous study involving rhamnolipid by De Jonghe et al. (2005) has underlined the necessity of re-application of biosurfactant after seven days in order to achieve effective control over the pathogen Phytophthora cryptogea in hydroponic system. In contrast, no additional application of the rhamnolipid BS was found to be necessary in this study. This may be attributed to induction of plant resistance as rhamnolipids have been reported to elicit defense responses and induce disease resistance. Varnier et al. (2009) demonstrated the role of rhamnolipids in triggering strong defense responses in grapevine which included early events of cell signaling like Ca²⁺ influx, reactive oxygen species (ROS) production and MAP kinase activation using Rha-C₁₀-C₁₀ and Rha-Rha-C₁₀-C₁₀ from P. aeruginosa and Rha-Rha-C₁₄-C₁₄ from Burkholderia plantarii. These rhamnolipids were also reported to induce a large battery of defense genes including some pathogenesis-related protein genes and genes involved in oxylipins and phytoalexins biosynthesis pathways.

The present study was successful in evaluating the antifungal activity of rhamnolipid against Fop using the susceptible cultivar Azad P-1. The use of 25 µg ml⁻¹ concentration of crude rhamnolipid caused complete disease inhibition. There was no negative effect of rhamnolipid on seed germination and fruiting in comparison to control plants. The rhamnolipid effectively caused complete disease inhibition in a single application during the course of the study. This may prove to be a decisive factor in successful implementation of the rhamnolipid as an antifungal agent against Fop.
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References


by heavy metal tolerant strain *Bacillus cereus* NK1. Colloids Surf B Biointerfaces 85:174–181

**Siddhartha Narayan Borah** is a research fellow at IASST, India. This research is a part of his PhD project on the antifungal activity of rhamnolipid against *Fusarium oxysporum* f. sp. *pisi*.

**Debahuti Goswami** is a research fellow at IASST, India. She is presently involved in production enhancement of biosurfactants and their application for crop disease control.

**Jiumoni Lahkar** is a research fellow at IASST, India. Currently she is carrying out research on application of biosurfactants for crop improvement.

**Hridip Kumar Sarma** is an assistant professor at the Department of Biotechnology, Gauhati University, Guwahati, India. His research specializes in environmental biotechnology and molecular microbial ecology.

**Mojibur Rahman Khan** is an assistant professor and Ramanujaswami fellow (DBT, Govt. of India). His research interests are environmental genomics and alternative energy.

**Suresh Deka** is a professor at the Life Sciences Division, IASST, India. His research interests include bioremediation of contaminated soil with plants and microbes, application of biosurfactants in agriculture and the petroleum industries.