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Isolation and characterisation of fusaricidin-type compound-producing strain of *Paenibacillus polymyxa* SQR-21 active against *Fusarium oxysporum* f.sp. *nevium*

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Abstract A bacterial strain was isolated from the rhizosphere of healthy watermelon plants in a heavily wilt-diseased field. This isolate was tentatively identified as *Paenibacillus polymyxa* (SQR-21) based on biochemical tests and partial 16S rRNA sequence similarity. The purified antifungal compounds were members of the fusaricidin group of cyclic depsipeptides having molecular masses of 883, 897, 947, and 961 Da with an unusual 15-guanidino-3-hydroxypentadecanoic acid moiety, bound to a free amino group. The strain SQR-21 was not able to produce antifungal volatile compounds but was able to produce cellulase, mannase, pectinase, protease, β-1,3-glucanase and lipase enzymes. However, the strain did not show any chitinase activity. Biocontrol potential of this strain was evaluated against *Fusarium oxysporum* cause of Fusarium wilt disease of watermelon in a greenhouse experiment. This strain combined with organic fertiliser decreased the disease incidence by 70% and increased the dry plant weight by 113% over the control.

Keywords Antifungal compound · Biocontrol potential · Disease incidence · Enzyme activity

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Introduction

The genus *Paenibacillus* (Ash et al. 1993); previously *Bacillus*; is facultatively anaerobic and belongs to endospore-forming low G+C Gram-positive bacilli. Among these, *Paenibacillus polymyxa* strains, formerly *Bacillus polymyxa*, reclassified by Ash et al. (1993), the type species of the genus *Paenibacillus,* is capable of suppressing plant diseases and promoting plant growth (Raza et al. 2008). *Paenibacillus polymyxa* strains are known to produce two types of peptide antibiotics. One group is active against bacteria including the polymyxins, polypeptins, jolipeptin, gavaserin, and saltavalin. The other group is made up of the peptide antibiotics active against fungi, Gram-positive bacteria and actinomycetes and includes the gatavalin antifungal compounds named as LI-F03, LI-F04, LI-F05, LI-F07, and LIF08, and fusaricidins A, B, C, and D (Beatty and Jensen 2002; Raza et al. 2008). In addition, there are many reports where the nature of the inhibitory agent is undefined (Seldin et al. 1999). *Paenibacillus polymyxa* spores because of sporangium deformation and thick walls can remain in a dormant state for long periods, being resistant to heat, drying, radiation and toxic chemicals (Comas-Riu and Vives-Rego 2002). *Paenibacillus polymyxa* strains are capable of producing several hydrolytic enzymes, including proteases, β-1,3-glucanases, cellulases, xylanase, lipase, amylase and chitinases which play an important role in the biocontrol of plant pathogens (Beatty and Jensen 2002; Parry et al. 1983; Raza et al.

2008). In addition, nitrogenase from *P. polymyxa* was separated into its component proteins and the *nifH* gene detected (Ding et al. 2005). *Paenibacillus polymyxa* strains are one of the best P-solubilising microbes isolated from the rhizosphere of canola plants and increase plant height and pod yield (De Freitas et al. 1997). Lebuhn et al. (1997) reported the production of auxin and other indolic and phenolic compounds by *P. polymyxa* strains. This growth-promoting and antagonistic potential is the base for effective applications of *P. polymyxa* strains as an alternative to the chemical control against a wide set of fungal and bacterial plant pathogens (Zhang et al. 2008).

Fusarium wilt of watermelon is caused by *Fusarium oxysporum* f.sp. *niveum* (FO) (Booth 1971) and this fungus also causes damping-off, cortical rot and the stunting of watermelon seedlings (Booth 1971). It is considered the most important soil-borne facultative pathogen, causing economically important losses of watermelon crop and limiting watermelon production in many areas of the world (Martyn 1996). Chemical fungicides and soil fumigators have been used to control Fusarium wilt of watermelon. However, dramatic increases in the resistance of the pathogen to chemicals and the toxicity and accumulation of the chemicals in the environment have prompted interest in alternative forms of disease control (Cook 1993). As an alternative, compost prepared from vegetable and animal market wastes, grape marc wastes, extracted olive press cake, sewage sludge and yard wastes has been demonstrated to be effective in the suppression of Fusarium wilt of tomato caused by *F. oxysporum* f. sp. *lycopersicci* race 1 (Trillas et al. 2006). In addition, *P. polymyxa* strains have been successfully used to control seedling blight, wilt and root rot of cucumber and water melon (Yang et al. 2004). However, co-application of composted material and antagonistic bacteria has been proved to be much more effective than the application alone of composted material and antagonistic bacteria (Zhang et al. 2008). In this study, we report the isolation and identification of a soil bacterium *P. polymyxa* and its effective use, in combination with organic fertiliser, to control Fusarium wilt. In addition, purification and identification of antifungal compounds from strain SQR-21 and its hydrolytic activity are also reported. The strain SQR-21 was found to be a chitinase deficient strain.

Materials and methods

Bacterial and fungal cultures

The *P. polymyxa* strain (SQR-21) was isolated by dilution plate techniques from the rhizosphere soil of healthy watermelon plants from a heavily wilt-diseased field. The cultures were maintained on Luria Bertani medium (LB) plates. The pathogenic strain of FO and the strain of *Bacillus circulans* were provided by the Soil-Microbe-Interaction Laboratory, Nanjing Agricultural University, Nanjing, China. The fungal pathogenic strain was maintained on PDA plates for 3 days at 28°C and the plates were then stored at 4°C. The pathogen was subcultured onto a fresh PDA plate after 3 months. The *P. polymyxa* SQR-21 and *B. circulans* strains were maintained on tryptic soya agar (TSA) plates and stored at −80°C in tryptic soya broth (TSB) + 20% glycerol for further use.

Identification of *Paenibacillus polymyxa* SQR-21

In addition to the biochemical properties of SQR-21 as mentioned in Table 1, salt tolerance was monitored at 2, 5, 7 and 10% of NaCl in TSB (30°C for 3 days). The morphological characters were observed on PDA plates and under the microscope (Olympus CX21, Olympus America Inc.). 16S rRNA gene was amplified directly from bacterial cells. The reaction mixture contained a small quantitiy of *P. polymyxa* cells pregrown overnight in LB medium, 2 μl each of reverse and forward primers (30 pmol μl^{-1}), 4 μl of dNTP mix, 5 μl of 10× PCR buffer containing 0.75 μl of 0.1 M $MgCl_2$, 32 μl of ddH_2O, 4 μl Tween-80 (10%) and 1 μl of *Taq* DNA polymerase (5 U μl^{-1}). The PCR conditions were as follows**:** 1 cycle for 5 min at 95°C and then 30 cycles for 1 min at 94°C, 1 min at 50°C and 1 min at 72°C and finally 15 min at 72°C. The amplified 16S rRNA was purified using a commercial DNA extraction kit (QIAGEN), ligated to pMD18-T vector (TaKa-Ra, Dalian), and transformed into *E. coli* DH5α cells via electroporation. The recombinant plasmid was prepared from a 5 ml overnight culture in LB medium using silica spin columns (QIAprep Spin Miniprep kit; QIAGEN) and sent for sequencing (Invitrogen™, Shanghai). The derived 16S rRNA gene sequence was compared to known bacterial sequences in NCBI GenBank using BLAST. Only results from the highest score queries

Table 1 Biochemical test results and different substrate utilisation of *Paenibacillus polymyxa* SQR-21 compared with *P. polymyxa* type-strain

Characteristic or test	Isolate SQR-21	*Paenibacillus polymyxa*[a]
Voges-Proskauer test	+	+
Amylase test	+, clear zones	+, clear zones
Citrate utilisation	–	–
Milk hydrolysis	+	+
Nitrate reduction	+	+
Indole production	–	–
Glucose fermentation	+	+
Arabinose; C source	+	+
Mannitol; C source	+	+
Glucose, lactose, sucrose	+, gas produced from glucose and lactose	+, gas produced from glucose
Triple sugar iron slant	+ anaerobic growth	+ anaerobic growth
Chitinase test	–	+

[a] The results of these tests on known *Paenibacillus polymyxa* strain are from Holt et al. (1994)

were considered for phylotype identification, with 98% minimum similarity. The 16S rRNA sequence has been submitted to GenBank with Accession Number: FJ600406.

Antifungal activity assay and optimisation of antifungal compound production

The antifungal activity was monitored on PDA plates by placing a 5 mm agar plug of fungus in the middle of the plate and by inoculating SQR-21 between the edge of plate and plug of fungus. After 5 days incubation at 28°C, the inhibition zone was measured. For the agar diffusion assay, a 5 mm agar plug of fungus was placed in the middle of the PDA plate and after 3 days, a 5 mm agar plug, 2 cm apart from edge of fungal growth, was removed and the hole was filled with 60 µl of antifungal material. Inhibition zone was measured after 2 days. For optimisation of antifungal compound production different media were tested such as potato dextrose broth (Mac Faddin 1985), nutrient broth (Marshall 1993), TSB (McCullough 1949), *Bacillus polymyxa* starch medium (http://www.nbimcc.org/cabricat/media.php), tryptone dextrose yeast extract medium (Vanderzant and Splittstoesser 1992), Katznelson minimal medium (Katznelson and Lochhead 1944) and King's B modified medium (Atlas 1995). For the selection of the best extractant, SQR-21 was incubated in King's B modified medium (200 ml) for 4 days and extracted twice with an equal volume (200 ml each) of four extractants; toluene, hexane, ethyl acetate and n-butanol. Among these, King's B modified medium and n-butanol was found to be the best so these were used in subsequent experiments. King's B modified medium was supplemented with different carbon sources (D-sucrose, D-maltose, D-glucose, D-ribose, D-fructose, glycerol, D-galactose, D-lactose, D-mannose and starch; 10 g l^{-1}) to optimise antifungal compound production. Antifungal compound production was also determined at different temperatures from 10–50°C with a difference of 5°C. Effect of incubation time on cell density and antifungal compound production was determined by incubating SQR-21 in King's B modified medium for 8 days at 30°C. Every day, optical cell density (660 nm) and antifungal activity was determined by the disc diffusion method.

Isolation and purification of antifungal compounds

For the isolation of antifungal compounds, King's B modified medium was inoculated with 1 ml of overnight culture of SQR 21 in TSB and incubated in an incubator shaker (170 rpm, 30°C). After 4 days, liquid culture was centrifuged at 12,000 *g* for 10 min and supernatants pooled together. Active compounds were extracted twice with an equal volume of n-butanol. The extract was concentrated by using a rotary evaporator and the residues were dissolved in methanol. The active compounds were eluted with

chloroform and methanol (50: 50) by using a silica gel column (2 cm×24 cm). Twenty-six fractions (10 ml each) were collected, dried by rotary evaporator and dissolved in 1 ml methanol. Active fractions, identified by agar diffusion assay against *F. oxysporum* on PDA, were pooled. These active fractions were further purified by a Sephadex LH-20 column (2 cm ×24 cm) equilibrated and eluted with methanol. Active fractions located by bioassay were pooled. Antifungal substances from Sephadex LH-20 were purified further with reverse-phase high performance liquid chromatography (Ettan™ LC, GE Healthcare Life Sciences) by using a Grace Vydac C18 238TP52 (5 μm) column and eluted in 5 to 95% of gradient B (0.05% formic acid in acetonitrile) in 80 min, with a flow rate of 0.2 ml min^{-1}; gradient A was 0.05% formic acid. The column fractions were monitored for absorbance at 214 nm (no absorbance at 280 was observed). After the agar diffusion assay against *F. oxysporum* on PDA, active peaks were located.

Mass spectrometry

Antifungal material was subjected to liquid chromatography-mass spectroscopic analyses (LC-MS) (Agilent 1100 HPLC-1946A MSP; Agilent Technologies, Santa Clara, USA) for molecular weight determination. The MS analysis was done by electrospray ionisation in positive ion mode. For further structural information, matrix-assisted laser desorption ionisation—time of flight (MALDI-TOF) MS treatment to antifungal material was done. The samples were prepared by using α-cyano-4-hydroxycinnaminic acid as a matrix on the surface of the gold target plate and the collision-induced generated ions were analysed by post-source decay (PSD) in reflectron mode with a TOF mass analyser. (ultraflex III, Bruker Daltonics Inc. USA). The antifungal materials were subjected to partial acid hydrolysis (3 N HCl, 100°C, 3 h) to obtain constituted amino acid information by Edman degradation.

Sensitivity to temperature, pH and degradative enzymes

For the thermal stability test, Sephadex LH-20 purified antifungal compound was exposed to 40, 60, 80 and 100°C for 1 h, 120°C for 30 min and 4 and −21°C for 1 week. For the pH stability test, antifungal compounds were solubilised in 100 mM citrate/phosphate buffer (pH 3–9), followed by incubation at 25°C for 2 h. The residual antimicrobial activity was assessed after neutralising the samples to pH 6.5 with an appropriate weak base or weak acid. Sensitivity of the antifungal compounds to various degradative enzymes was tested. Enzymes tested were amylase (26.8 U mg^{-1}; Jiangyin Bsdzymer bioengeineering, China), lipase from Porcine pancreas (≥3 U mg^{-1}; 64005732; SCRC, China), protease from *Streptomyces griseus* (5.2 U mg^{-1}; P5147; Sigma-aldrich), proteinase K recombinant (600–100 U mg^{-1}; 03115828001; Roche Applied Science) and trypsin from bovine pancreas (≥2500 U mg^{-1}; 64008834; SCRS, China). All enzyme solutions were prepared in 25 mM phosphate buffer (pH 7.0), each containing 1 mg ml^{-1} except lipase solution, which contained 0.1 mg ml^{-1}. Solutions of the antimicrobial peptide were prepared in the same buffer. The stock solutions of antifungal compound and enzymes were mixed at a 1:1 ratio (vol/vol) and incubated at 37°C for 1 h before residual antimicrobial activity measurement by agar diffusion assay against *F. oxysporum*.

Antifungal volatile compounds assay

The strain *P. polymyxa* was streaked on PDA and nutrient agar medium (NA) in the Petri plates separately. A 5 mm mycelial plug was cut from the margin of an actively growing culture of *F. oxysporum* and placed in the centre of a second Petri plate containing PDA. The dish containing the mycelial plug was inverted over the bacterial plates and the dishes were sealed with Parafilm and incubated at 28°C. The diameter of fungal mycelium was measured every 24 h, and compared to the control, over a period of 7 days. The control plates had mycelial discs only. There were four replicates for each treatment and the experiments were repeated twice.

Hydrolytic activity test

For cellulase, pectinase and mannase activity, M9 medium agar (Miller 1974) was amended with 10 g l^{-1} each of cellulose, mannose and pectin separately and 1.2 g l^{-1} of yeast extract, in which a clear halo around the colonies after 7 days of incubation at 30°C was considered as positive for

cellulase and mannase production. For determining pectinase, the plates were flooded with 2 M HCl after 2 days of incubation at 30°C. Clear halos around the colonies were considered as positive for pectinase production (Cattelan et al. 1999). Lipase and proteolytic activity were determined by inoculating lipase agar medium and skimmed milk agar medium, respectively, with SQR-21. After 2 days of incubation at 30°C, clear zone around the colonies indicated positive activity (Smibert and Krieg 1994). *Bacillus circulans* strain was used as positive control for all enzyme assays. Each experiment was done in three replicates.

Colloidal chitin was prepared from commercial chitin (Roberts and Selitrennikoff 1988). Qualitative chitinase test was performed by amending M9 minimal agar medium with 0.5% colloidal chitin and 2 g l^{-1} peptone, in which a clear halo around the colonies after 7 days of incubation at 30°C was considered as positive for chitinase production. For the quantitative test, 5 ml of precultured SQR-21 overnight in TSB was inoculated into 200 ml of the M9 medium, amended with 0.3% colloidal chitin and 2 g l^{-1} of peptone and further incubated for 7 days at 30°C. The broth culture was centrifuged at 12,000 *g* for 10 min at 4°C. The proteins from the supernatant were precipitated with $(NH_4)_2SO_4$ (80% saturation), centrifuged and dissolved in a small volume of sterile distilled water. The solution was dialysed overnight at 4°C against four changes of 4 l each of distilled water containing 1 mM EDTA and used to determine both chitinase and β-1,3-glucanase activity. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of NAG min^{-1} (Wiwat et al. 1999). *Bacillus circulans* strain was used as the positive control. The experiment was repeated three times. The β-1,3-glucanase activity was determined qualitatively by using laminarin (1 mg ml^{-1}) as a substrate according to the Nelson-Somogyi method (Nelson 1955; Somogyi 1945) . Blue colour indicated positive reaction. Proteins were determined by the method described by Bradford (1976).

Soil, watermelon cultivar and bio-organic fertiliser

Sandy loam soil was collected from the surface of protected cultivation plots with a 5-year history of continuous watermelon cultivation that suffered from severe wilt disease, in Shanghai, China. Seeds of watermelon *Citrullus lanatus*. And Nakai cv. Zaojia 84–24 were obtained from the Nanjing Vegetable and Horticulture Research Institute, Nanjing, China. Organic fertiliser was made in the laboratory as follows. Organic fertiliser used for the Bio-product was composed of amino acid fertiliser and pig manure compost. Amino acid fertiliser was made from oil rapeseed cakes enzymatically hydrolysed by aerobic microbial fermentation at <50°C for 7 days to obtain amino acid fertiliser. This amino acid fertiliser contained 44.2% organic matter and 12.9% amino acids, small molecular peptides and oligo peptides. The nutrient content was 4.4% N, 3.5% P_2O_5, and 0.67% K_2O. Pig manure compost was made by Tianniang Ltd. in Suzhou, China by composting pig manure at a temperature range of 30-70°C for 25 days. This compost contained 30.4% organic matter, 2.01% N, 3.7% P_2O_5, and 1.1% K_2O. The antagonistic microbe was prepared as follows: *P. polymyxa* strain SQR-21 was incubated in a beef extract and peptone liquid culture on a shaker at 170 rpm at 37°C for 2–3 days. The known amount of the culture of *P. polymyxa* (CFU was determined prior to addition to the organic fertiliser using a hemocytometer) was added and mixed with a known dried weight (DW) of organic fertiliser so as to obtain a final concentration of the strain in the bioorganic fertiliser (BOF) of about 3×10^9 CFU g^{-1} DW BOF of *P. polymyxa*.

Pot experiment and data collection

Six treatments in the greenhouse experiment were designed: (1) control (Fusarium-infested field soil only); (2) CF (Fusarium-infested field soil added with chemical fertilisers containing equal amounts of N as urea and P and K as KH_2PO_4, as in 0.1% OF (w/w)); (3) 0.1% OF (Fusarium-infested field soil supplemented with 0.1% OF); (4) 0.5% OF (soil supplemented with 0.5% OF); (5) 0.1% BOF (Fusarium-infested field soil supplemented with 0.1% BOF); (6) 0.5% BOF (Fusarium-infested field soil supplemented with 0.5% BOF). Each treatment had five replicates (five pots). All fertilisers were mixed thoroughly with soil to ensure an even distribution of fertilisers and microbes. Each pot 20 cm diam, height 25 cm) was filled with 5 kg of Fusarium-infested field soil (wet weight) and planted with ten seedlings. Two weeks later, eight seedlings were adjusted for the experiment. All pots were arranged randomly. Plants were

grown at a maximum of 35°C (day) and minimum of 21°C (night) at a relative humidity of 60–85% under daylight conditions in a greenhouse of Nanjing Agricultural University, China. The wilt incidence was determined 63 days after the experiment was carried out in a greenhouse. Disease incidence was expressed as the percentage of diseased plants over the total number of plants. For the determination of dry plant weight, two plants were sampled from each pot, the roots were washed thoroughly, blot-dried and the plants dried at 70°C until constant weights.

Statistical analysis

The values of wilt percentage were represented as the means of five replicates (mean±SE) from five pots. The arcsin conversion was done before analysing the data for percentages. Differences were assessed with one-way ANOVA. Duncan's multiple-range test was applied when one-way ANOVA revealed significant differences ($P \leq 0.05$). All statistical analysis was performed with SPSS BASE ver.11.5 statistical software (SPSS, Chicago, IL).

Results

Identification of SQR-21

Watermelon rhizosphere soil was used to isolate microorganisms with antifungal properties and SQR-21 was selected as one such isolate with particularly good antifungal activity against *F. oxysporum*. SQR-21 cells were motile, Gram-positive rods approximately 2–4×1–1.5 μm. When grown on PDA medium, SQR-21 colonies were opaque, tan-coloured and glistening. Biochemical test (Table 1) results regarding acid and gas production by carbohydrate utilisation showed that SQR-21 was not able to utilise inositol and sorbitol. Acid and gas production was observed by utilisation of lactose, glucose and mannitol while all other carbohydrate utilisation showed positive reaction only for acid production. The salt tolerance test results showed that SQR-21 was not able to grow above 2% NaCl concentration in TSB. In addition, a 1497 bp region of the 16S rDNA sequence of SQR-21 was compared with that of known species of microorganisms (Fig. 1) and the strain was identified as *P. polymyxa*.

Optimisation of antifungal compound production

To determine the effect of incubation time on growth and antifungal compound production in King's B modified medium, the strain SQR-21 was incubated for 8 days as described in the Materials and methods section. Maximum growth was obtained on the second day while increase in antifungal activity was observed from the third to sixth days, and then growth and antifungal activity decreased gradually up to the eighth day (Fig. 2). Furthermore, King's B modified medium was supplemented with various carbon sources to optimise antifungal activity and the medium containing glycerol, ribose and lactose showed maximum antifungal activity (Fig. 2). Growth and antifungal activity of SQR-21 was found to be optimal at 30 to 35°C. Therefore, a temperature of 30°C was maintained in all subsequent studies, but growth and production of antifungal activity was noted to persist at temperatures as low as 15°C (Fig. 2).

Characteristics of antifungal compounds

After the final purification with RP-HPLC, antifungal activity was observed in two eluted fractions, one eluted at 38.4 min and the second at 39.9 min (Fig. 3). LC-MS analysis results showed that each peak was comprised of two compounds; the first had molecular weights of 947.5 and 961.5 Da $(M+H)^+$ (Fig. 3) while the second had molecular weights of 883.5 and 897.5 Da $(M+H)^+$ (Fig. 3). A variety of chromatographic conditions and mobile phases were tested in an attempt to separate these components, but further resolution could not be obtained. Further structural information was obtained by using MALDI-TOF analysis. The MALDI-TOF mass spectrum of the 883-Da molecular ion showed a peak at 256 Da as well as a peak at 628 Da (the difference between the 883-Da molecular ion and the 256-Da fragment). The 256 Da ion peak was interpreted to represent the 15-guanidino-3-hydroxypentadecanoic acid (GHPD) side chain that was easily fragmented from the cyclic peptide and the 628 Da ion peak comprised the remaining cyclic ring structure of the peptide. Amino acid sequence information was not obtained from MALDI-TOF analysis. In the same way, other compounds were also subjected to MALDI-TOF analysis and all showed the presence of the 256 Da ion peak with remaining peaks of the amino acid ring

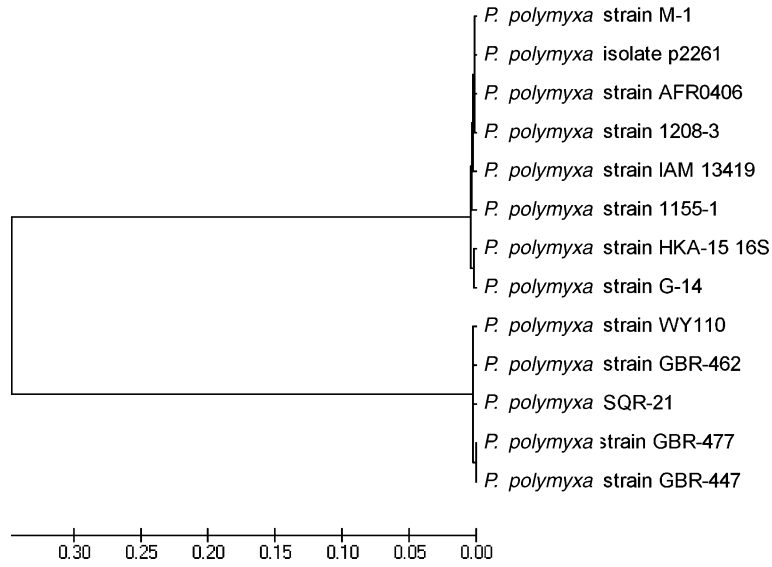

Fig. 1 Phylogentic tree derived from GenBank based on *Paenibacillus polymyxa* SQR-21 16SrDNA sequence

and its fragmentation information. For gathering information about the amino acids, antifungal material was subjected to Edman degradation but information was not achieved. When antifungal material was subjected to acid hydrolysis and then to Edman degradation, amino acid information was obtained. This analysis indicated that the following amino acids constitute the first peak; threonine, valine, tyrosine, asparagine, glutamine and alanine and the second peak was constituted by threonine, valine, asparagine, glutamine and alanine. An unknown amino acid that did not correspond to any of the 20 protein amino acids was also observed in all compounds. Considering the literature and our results, the first peak compounds having molecular weights of 947.5 and 961.5 Da $(M+H)^+$ were designated as Fusaricidin C and Fusaricidin D, respectively while second peak compounds having molecular weights of 883.5 and 897.5 Da $(M+H)^+$ were designated as Fusaricidin A and Fusaricidin B, respectively. The antifungal material was soluble in methanol, n-butanol, ethanol, acetonitrile, isopropanol and 0.1% acetic acid, slightly soluble in water, n-hexane and ethyl acetate and insoluble in toluene and methylene chloride. The enzymes amylase, lipase, protease, proteinase K and trypsin were unable to degrade antifungal compounds and were stable at all temperatures (−21, 4, 40, 60, 80, 100 and 120°C) and a pH range of 2–10.

Antifungal volatile compounds assay

The results showed that under the experimental conditions, *P. polymyxa* was not able to produce any such volatile compound that can inhibit the growth of *F. oxysporum* in both PDA and NA media as compared with the control.

Hydrolytic activity test

Qualitative assays of hydrolytic enzymes showed that the strain SQR-21 was able to produce all tested hydrolytic enzymes such as cellulase, mannase, pectinase, protease, lipase and β-1,3-glucanase. Chitinase activity was determined qualitatively and quantitatively. On agar plates amended with 0.5% colloidal chitin, no clear zone was observed up to 7 days of incubation, but in the control it was very clear. In liquid culture, the strain SQR-21 was unable to degrade colloidal chitin even after 7 days of incubation while the positive control strain *B. circulans* degraded almost all colloidal chitin. In addition, both cell-free liquid culture of SQR-21 and precipitated proteins were unable to degrade the reducing end group, N-acetylglucosamine (NAG) from colloidal chitin. However growth and precipitated proteins (158 mg) were enough to determine β-1,3-glucanase activity in SQR-21. In cell-free liquid culture of the positive control strain *B. circulans*, total

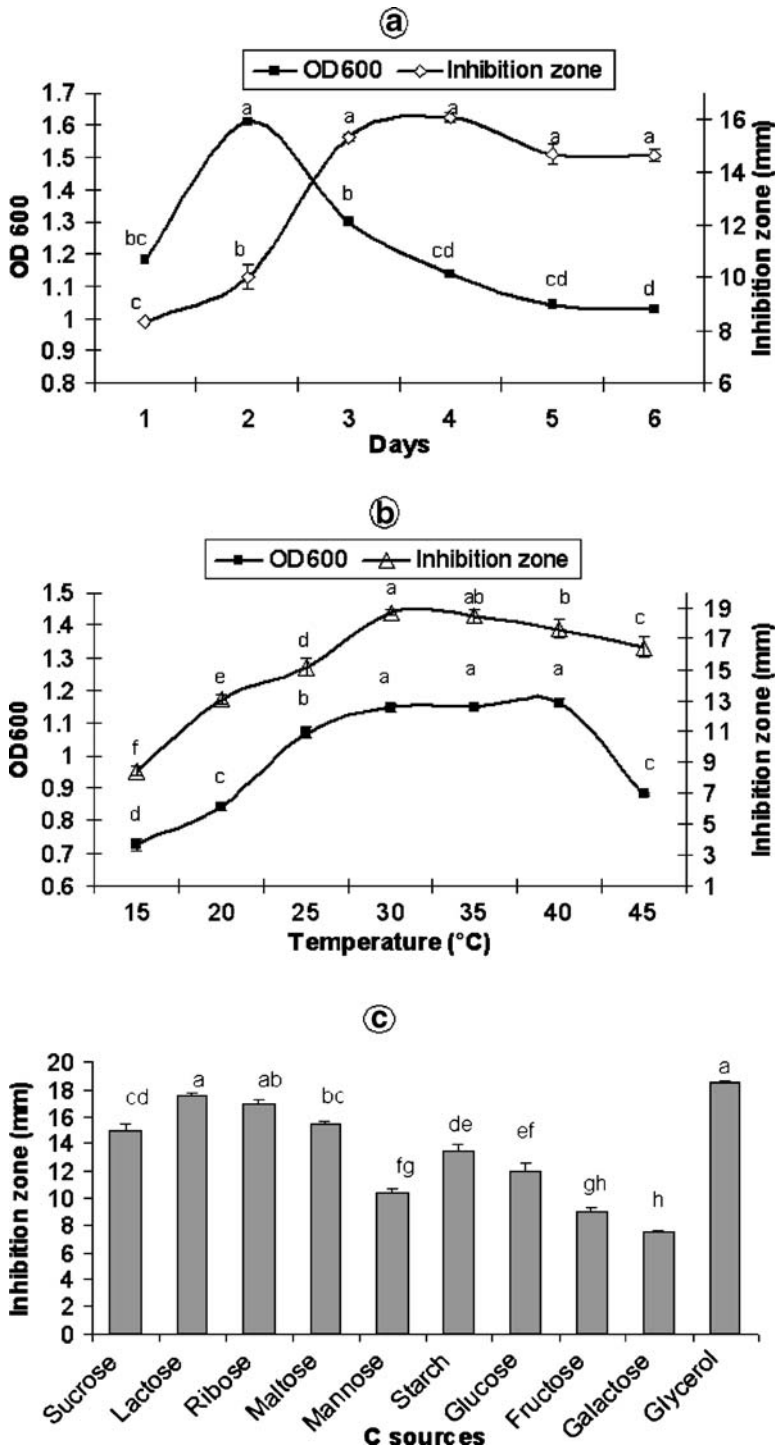

Fig. 2 Growth and antibiotic production by *Paenibacillus polymyxa* SQR-21 in King's B modified medium. **a**, effect of different temperatures on growth and antibiotic production **b** and the effect of different C sources on antifungal compound production **c**; Antifungal antibiotic production was determined by bioassaying n-butanol extracts of liquid culture

protein and total and specific chitinase activity were 800 mg, 155 U and 0.19 U mg^{-1}, respectively, while in precipitated proteins of *B. circulans* these were 137 mg, 30 U and 0.21 U mg^{-1}, respectively.

Dry weight and disease incidence rate

The bioorganic fertiliser prepared in our laboratory significantly inhibited Fusarium wilt of watermelon

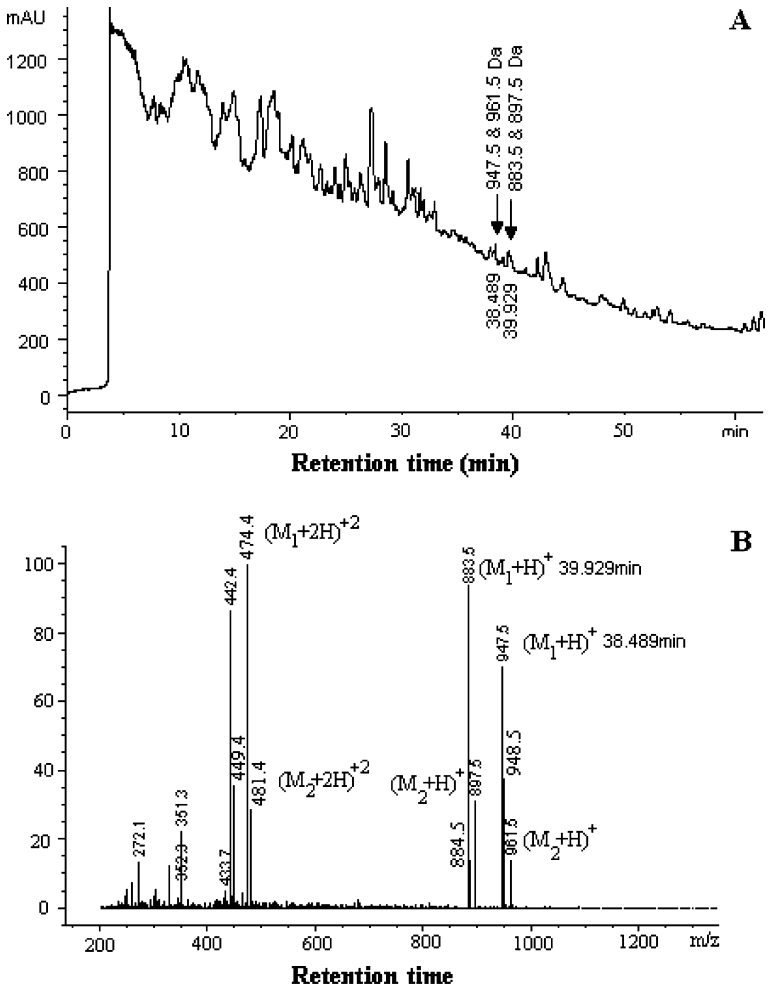

Fig. 3 RP-HPLC peak profile of antifungal material from *Paenibacillus polymyxa* SQR-21. Antifungal activity was found in peaks eluted at 38.4 and 39.9 min, as indicated by the arrowheads (**a**). Mass spectroscopic analysis of antifungal compounds, produced by *P. polymyxa* SQR-21, showing molecular weights (947.5 and 961.5 Da) and (883.5 and 897.5 Da) of active peaks that showed antifungal activity. Mass spectroscopy was done in positive ion mode (**b**)

in the greenhouse experiments. Incidence rates in the control and CF (chemical fertiliser) treatments were higher than in the other treatments. The incidence rate was the lowest in the treatment with 0.5% BOF, with a decrease of 70% compared with the control (Fig. 4). The BOF not only suppressed Fusarium wilt but also significantly promoted watermelon growth and thus increased plant dry weight. The lowest dry weight was found in the control treatment plants, while the highest was found in plants treated with 0.5% BOF (increment of 113% in comparison with the control). The dry weight of plants in all bioorganic fertiliser-related treatments was higher than that in the control plants (untreated) in a rate-dependent manner (Fig. 4).

Discussion

A newly isolated chitinase-deficient strain was identified as *P. polymyxa* SQR-21 based on 16 S rDNA sequence information and cellular and colony morphology that were similar to that described for the type culture of *P. polymyxa* ATCC 842 (Parry et al. 1983). The strain SQR-21 was originally isolated by cultivating it on PDA where the inhibition of

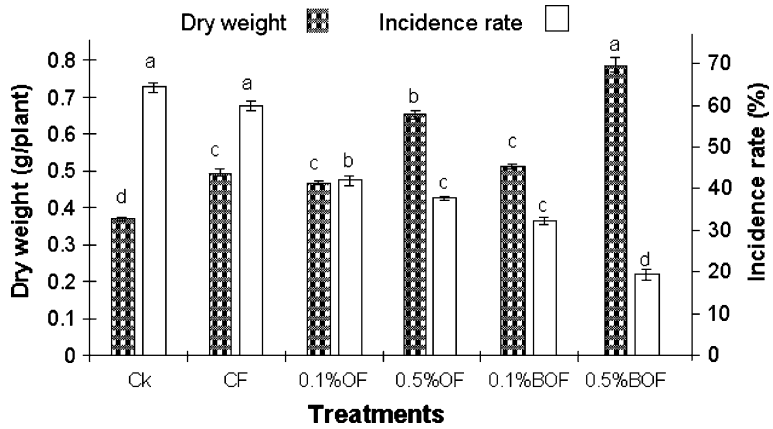

Fig. 4 Incidence rate of Fusarium wilt and dry weights of watermelon treated with organic fertiliser and bioorganic fertiliser. All values are the means of five pots. Columns with the same letter are not significantly different at $P=0.05$ according to post-hoc tests (Duncan's LSD test); each mean was compared with each of the other means. Bars indicate the standard error of the mean

F. oxysporum growth was significant. PDA has a pH of about 5 and is used frequently for the cultivation of fungi, but is too acidic for optimal growth of most bacteria. Therefore, to optimise growth and antifungal activity, different media were tested and maximum antifungal activity was found in King's B modified medium although it did not support maximum growth. This medium has glycerol as the carbon source that might inhibit growth but supported antifungal compound production. The strain SQR-21 was found to grow well at 30°C but it also showed growth and antifungal compound production at 15°C. This more closely approximates soil temperatures found in cucumber and watermelon-growing regions and holds promise for the use of SQR-21 as a biocontrol agent (Beatty and Jensen 2002).

Beatty and Jensen (2002) reported that the antifungal activity produced by *P. polymyxa* PKB1 was associated with spores harvested in the early stationary phase of growth. No antifungal activity could be recovered from the culture broth and methanol was used to extract antifungal compounds from harvested spores. We used n-butanol to extract antifungal compounds from cell-free liquid culture of SQR-21. The digestion of heat and pH-stable antifungal antibiotics with different enzymes showed no reduction of antifungal activity. It suggested that if it is a peptide, it must be cyclic: N- and C-terminally blocked or composed of unnatural amino acids. Cyclic peptides can be resistant to cleavage by proteases because their cyclic structure renders them relatively inflexible, which may make cleavage sites inaccessible because of steric hindrance (Eckart 1994). These peptides often owe their cyclic nature to the presence of ester linkages and are then referred to as cyclic depsipeptides (Beatty and Jensen 2002). The antifungal material was subjected to Edman degradation but N-terminal sequence information was not achieved; in addition, MS-MS analysis also gave just partial fragment information, again suggesting that the peptide was blocked at the N-terminus or cyclised.

A *P. polymyxa* strain, *P. polymyxa* ssp. *colistinus koyama* produced an antifungal compound called gatavalin. Mass spectroscopic analysis indicated that the material was an insepartable mixture of two peptides with masses of 883 and 897 Da (Nakajima et al. 1972). The close agreement in masses suggested that SQR-21 might be producing a form of a gatavalin-like peptide. However, Beatty and Jensen (2002) reported the same HPLC profile and molecular weights of gatavalin-like peptides produced by *P. polymyxa* ssp. *colistinus koyama* and fusaricidin-like peptides produced by *P. polymyxa* PKB1. This provides a strong indication that *P. polymyxa* ssp. *colistinus koyama, P. polymyxa* PKB1 and *P. polymyxa* SQR-21 produced the same or a similar family of peptide antibiotics. However, no further structural information has since appeared. Another strain of *P. polymyxa* produces a series of antifungal peptides that are designated as LIF03, LI-F04, LI-F05, LI-F07, and LI-F08. Once again, no full

structural analyses were conducted, but mass spectroscopic analyses indicated that LI-F04, one of the major products, was actually a mixture of two peptides with masses of 883 and 897 Da and LI-F03 was a mixture of two peptides with masses 947 and 961 Da (Kurusu et al. 1987). The isolation of another strain of *P. polymyxa* was reported that produced peptide antibiotics designated as fusaricidins A, B, C and D having masses of 883, 897, 947 and 961 Da, respectively (Kajimura and Kaneda 1996). The MALDI-TOF mass spectrum of our antifungal compounds showed the presence of a 256 Da ion peak with remaining peaks of amino acid ring and its partial fragmentation information. The 256 Da ion peak was interpreted to represent the 15-guanidino-3-hydroxypentadecanoic acid (GHPD) side chain that was easily fragmented from the cyclic peptides. For gathering information about the amino acids, antifungal material was subjected to acid hydrolysis and then to Edman degradation. Beatty and Jensen (2002) reported similar earlier results for fusaricidin-like peptides.

Comparing our results with reported results (Beatty and Jensen 2002; Kajimura and Kaneda 1996; Kurusu et al. 1987; Nakajima et al. 1972) it was concluded that the strain *P. polymyxa* SQR-21 produced cylic lipopetide fusaricidin-type antifungal compounds named fusaricidin A, B, C and D. Fusaricidins A and B differ only in a single amino acid residue (A has an asparagine residue in position 5, whereas B has a glutamine residue) to give masses of 883 and 897 Da, respectively. In the same way, fusaricidin C and D differ only in a single amino acid residue (C has an asparagine residue in position 5, whereas D has a glutamine residue) to give masses of 947 and 961 Da, respectively. According to the literature, the unknown amino acid was D-allo-threonine (Kajimura and Kaneda 1996; Beatty and Jensen 2002).

The antifungal volatile compounds production by Bacillus species, such as *Bacillus subtilis*, has been reported (Chen et al. 2008). However, the strain SQR-21 was not able to produce any antifungal volatile compound in two media. Qualitative analysis of hydrolytic activity showed that the strain SQR-21 was able to produce all tested hydrolytic enzymes except chitinase. It also showed a positive reaction for nitrogenase and phosphatase. These findings are consistent with previous reports (Beatty and Jensen 2002; Parry et al. 1983; Raza et al. 2008). Many scientists have reported positive chitinase activity of *P. polymyxa* (Beatty and Jensen 2002; Parry et al. 1983). However, the strain SQR-21 did not show any chitinase activity on agar plates and in liquid culture although growth of SQR-21 was enough to detect β-1,3-glucanase activity. Waldeck et al. (2006) isolated two chitinase-deficient strains of *Bacillus licheniformis* where chitinase negative activity was detected due to the deletion of an A within the coding region of *chiA*. Further genetic information about chitinase activity of *P. polymyxa* is not yet available. Chitinase negative activity of *P. polymyxa* SQR-21 might be due to gene transfer or mutation caused by unknown sources.

We report here a new biocontrol product involving combinations of *P. polymyxa* SQR-21, an antagonistic bacterium with a special organic fertiliser fermented from oil rapeseed cake. The method combines the merits of the antagonistic microbe and a superior organic fertiliser, thereby inhibiting the pathogen and reducing the incidence of Fusarium wilt disease while also supplying sufficient nutrients for plant growth. A single fertiliser was able, by itself, to depress the incidence of Fusarium wilt of watermelon (34%) but the presence of *P. polymyxa* almost doubled the suppression of disease incidence (70%). The protection of the watermelon plant from the pathogen by the BOF was also reflected by the increase in dry weight (113%) of the watermelon plants. The chemical fertilisers gave the same dry weights of plants in comparison with 0.1% OF and 0.1% BOF; however, higher levels of OF and BOF (0.5%) increased the dry weights compared with the chemical fertiliser. This suggests that well-organised combinations of organic fertiliser with antagonistic strains in such a product and a reasonable application rate of the BOF could be useful in controlling Fusarium wilt of watermelon. The results are similar to those obtained for Fusarium wilt of banana, where soil application of *P. fluorescens* with neem cake gave the best results (Saravanan et al. 2003). The results are also in agreement with the significant suppression of rhizoctonia disease of cucumber by a mixture of agricultural compost and *Trichoderma* spp. (Trillas et al. 2006). Zhang et al. (2008) also observed an increase in dry weight of cucumber plants by the addition of organic fertiliser amended with *P. polymyxa*. Purification and characterisation of the

antifungal compounds (fusaricidin A, B, C and D) from *P. polymyxa* SQR-21 have helped to clarify the relationship between related groups of antifungal peptide antibiotics produced by various isolates of *P. polymyxa*.

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