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Antifungal potential of *Bacillus vallismortis* R2 against different phytopathogenic fungi

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Abstract

The cash crops grown in an agro-climatic region are prone to infection by various fungal pathogens. The use of chemical fungicides over the years has resulted in emergence of resistant fungal strains, thereby necessitating the development of effective and environmental friendly alternatives. The natural antagonistic interactions among different microbial populations have been exploited as an eco-friendly approach for controlling fungal pathogens resistant to synthetic chemicals. Morphologically distinct bacterial cultures (150), isolated from rhizospheric soils of wheat, rice, onion and tomato plants were screened for their antifungal potential against seven phytopathogenic fungi prevalent in the State of Punjab (India). The bacterial isolate R2, identified as *Bacillus vallismortis*, supported more than 50% inhibition of different phytopathogenic fungi (*Alternaria alternata*, *Rhizoctonia oryzae*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Colletotrichum* sp, *Helminthosporium* sp and *Magnaporthe grisea*) in dual culture plate assay. The thin layer chromatography based bio-autography of acid-precipitated biomolecules (APB) indicated the presence of more than one type of antifungal molecule, as evidenced from zones of inhibition against the respective fungal pathogen. The initial analytical studies indicated the presence of surfactin, iturin A and fengycin-like compounds in APB. The antifungal activity of whole cells and APB of isolate R2 was evaluated by light and scanning electron microscopy. The wheat grains treated with APB and exposed to spores of *A. alternata* showed resistance to the development of black point disease, thereby indicating the potential application of R2 and its biomolecules at field scale level.

Additional key words: rhizosphere; microscopy; black point disease; *Alternaria alternata*; bio-autography; biomolecules.

Abbreviations used: APB (acid-precipitated biomolecules); CLP (cyclic lipopeptide); HPLC (high performance liquid chromatography); MR (methyl red); SEM (scanning electron microscopy); TLC (thin layer chromatography); YME (yeast malt extract).

Citation: Kaur, P. K.; Kaur, J.; Saini, H. S. (2015). Antifungal potential of *Bacillus vallismortis* R2 against different phytopathogenic fungi. Spanish Journal of Agricultural Research, Volume 13, Issue 2, e1004, 11 pages. <http://dx.doi.org/10.5424/sjar/2015132-6620>.

Received: 31 Jul 2014. **Accepted:** 07 Apr 2015

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Funding: This work was supported by University Grant Commission, UGC [Grant F1-17.1/2010/MANF-SIK-PUN-1821/(SA-III/Website)].

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Agriculture is the mainstay of economy in the State of Punjab (India) with wheat (*Triticum aestivum*), rice (*Oryza sativa*), onion (*Allium cepa*) and tomato (*Lycopersicon esculentum*) as the major cash crops cultivated in the State. The infestation of these crops by fungal pathogens, i.e. *Alternaria alternata*, *Rhizoctonia oryzae*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Colletotrichum* sp, and *Magnaporthe grisea*, affects the overall yield thus causing significant economic losses to farmers (Mew, 1991; Roberts *et al.*, 2005; Zhao *et al.*, 2010). The black point or kernel smudge of wheat caused by different fungal pathogens, including *A. alternata*, has emerged

as a serious concern to wheat cultivation (Sisterna & Sarandon, 2000; Monaco *et al.*, 2004). This disease is prevalent in the States of Punjab and Haryana due to warm and dry weather conditions supporting the growth of fungal pathogens (Singh *et al.*, 2003). The disease adversely affects the average weight, germination rate and colour of grains thereby affecting the overall yield and quality of flour and its products (Dexter & Matsuo, 1982). The extensive use of chemical fungicides over the years has led to the development of resistant fungal strains, thereby necessitating the application of higher concentration of chemical fungicides, which in turn have detrimental effects on the ecosystem (Cakmakci, 2007). Thus, there is a need to develop environmental friendly

strategies to decrease the dependence on chemical fungicides and to address the problem of recurrent incidents of infestation by resistant fungal pathogens.

The studies over the last few years have reported the antifungal potential of biomolecules viz. lipopeptides, glycolipids, phospholipids, neutral lipids, polysaccharide-protein complexes and fatty acids produced by different bacterial isolates (El-Ghaouth, 1997; Szczech & Shoda, 2006; Romero *et al.*, 2007, Tendulkar *et al.*, 2007). The present study was taken up to isolate and screen the unexplored bacterial diversity of this region for its antifungal potential so as to develop biocontrol strategies as an alternative to chemical fungicides.

Material and methods

Phytopathogenic fungal strains and growth conditions

The fungal pathogens commonly causing disease to cash crops in the State of Punjab (India) viz. *A. alternata*, *R. oryzae*, *F. oxysporum*, *F. moniliforme*, *Colletotrichum* sp, *Helminthosporium* sp and *M. grisea* were obtained from the Department of Plant Pathology, Punjab Agricultural University (PAU), Ludhiana. The fungal strains were maintained on potato dextrose agar (PDA) medium by regular sub-culturing and preserved for long term at -80°C as 25% (v/v) glycerol stocks.

Isolation

The soil samples from rhizosphere —defined by Hiltner (1904) as the zone in which plant roots interact with the surrounding soil— of wheat, rice, onion, and tomato plants were collected from agricultural fields located in and around the city of Amritsar (Punjab). One gram of each soil sample was serially diluted in 9 mL sterile saline (0.8% w/v NaCl), and 0.1 mL of the appropriate dilution was plated on yeast malt extract (YME) agar medium. The plates were incubated at 30°C and observed daily for three days. Morphological distinct bacterial colonies were picked and repeatedly streaked to ascertain their purity.

Screening

The bacterial isolates were screened for their antifungal potential by dual culture plate assay. Fungal spores were inoculated on YME agar plates, which were incubated at 30°C for seven days. Agar plugs of 5 mm diameter of each fungus were taken from these plates and placed in the centre of the YME agar plates

having four different bacterial isolates streaked per plate, one isolate in each quarter. The plates were incubated at 30°C and observed daily up to seven days. The fungal growth in presence of bacterial isolates was compared with that of growth in absence of bacterial isolates (control) to determine the percentage of inhibition, using the formula: $(r_1 - r_2)/r_1 \times 100$, where r_1 and r_2 represent the radius (mm) of fungal growth in absence and presence of bacterial isolate, respectively.

On the basis of these observations, the isolate R2 was selected for further studies as it showed best antifungal potential among all the screened isolates.

Identification of isolate R2

The identification of isolate R2 was carried out by routine biochemical tests and by amplifying the sequence of gene coding for 16S rRNA using universal primers 27F and 1492R (Cappuccino & Sherman, 1999; Frank *et al.*, 2008). The sequence was aligned with already available sequences in GenBank database (NCBI) using ClustalW software version 1.81 (<http://www.clustal.org/clustal2/>). The phylogenetic tree was constructed by neighbor-joining method using MEGA software version 5.1 (<http://mega.software.informer.com/5.1b/>) to identify the isolate on basis of percentage homology.

Preparation of acid-precipitated biomolecules (APB)

One litre of YME broth in a 3 L Erlenmeyer flask was inoculated with 0.1% (v/v) overnight activated cells of R2. The flask was incubated for 48 h at 30°C in an orbital shaker (180 rpm). The broth was centrifuged at 10,000 rpm for 10 min and pH of the supernatant was lowered to 2.0 with concentrated HCl (11.6 N). This acidified supernatant was kept at 4°C for 24 h to allow complete precipitation. The APB obtained were pelleted by centrifugation at 10,000 rpm for 15 min in a pre-weighed centrifuge tube. The pellet was dried and dissolved in methanol to prepare 1% (w/v) stock solution. The stock solution was filter-sterilized (Acrodisc 13 mm with $0.2\ \mu\text{m}$ Supor Membrane, Pal Life Sciences, USA) and used for further studies.

Analysis of APB by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC)

The APB preparation was resolved on a TLC plate (silica gel 60 F₂₅₄, Merck, Germany) and compared with

the standards of iturin A and surfactin (Sigma, USA) using a mixture of water-methanol-chloroform (1/4/5 v/v) as developing solvent. The resolved components were visualized by exposing the TLC plate to iodine vapours. The plates were also sprayed with distilled water to ascertain the presence of lipopeptides as described by Li *et al.* (2007). The crude APB and standards of iturin A and surfactin (Sigma, USA) were further analyzed by HPLC using C18 reverse phase column (250 mm × 4.6 mm × 1/4 Microsorb VIV 100-5, Varian, USA) at a constant oven temperature of 30 °C (TCC-100, Dionex, USA). The sample (20 µL), was injected and eluted using solvent system of acetonitrile-water (70/30) containing 0.05% (v/v) trifluoroacetic acid, at a flow rate of 1 mL/min. The eluant was read at 225 nm using photo diode array detector (UVD340U, Dionex, USA).

TLC agar overlay assay

The TLC-based bio-autographs were developed as described by Kaur *et al.* (2006). The YME agar supplemented with 10⁴ spores/mL of the respective test fungus was poured on TLC plates with resolved APB. The TLC plate developed without APB in solvent system as described above and overlaid with YME agar containing spores of respective fungus served as control. The TLC plates were placed in 200 mm glass Petri dishes and incubated at 30 °C to observe the clear zones or variation in hyphal growth pattern.

Microscopic evaluation of antifungal potential of R2 cells and their APB on *A. alternata* growth and morphology

The variation in morphology of *A. alternata* grown in presence and absence of cells and APB of R2 was observed by light microscopy and scanning electron microscopy (SEM). In light microscopy studies, an agar plug of *A. alternata* taken from well grown lawn of seven days old fungus was kept in the centre of fresh YME agar plate having four overlapping streaks of R2 cells, each at a distance of 1.5 cm from circumference of the plate. The control plate had fungal agar plug in the centre without any bacterial streaks. The plates were incubated for seven days at 30 °C and observations related to size and appearance of fungal growth were recorded daily. The hyphal growth on respective plate was observed using inverted microscope (Olympus CKX41, Japan).

The effect of whole cells of R2 and APB on growth of *A. alternata* was evaluated by shake flask studies.

The cells of R2 grown overnight in YME broth were pelleted and washed with 0.8% (w/v) saline. The pellet was suspended in 0.8% (w/v) saline to achieve an OD₆₀₀ of 0.68, equivalent to 10⁸ CFU/mL. The cells of R2 and spores of *A. alternata* (10⁴ spores/mL) were inoculated in 20 mL YME broth in 100 mL Erlenmeyer flask to evaluate the antagonistic effect of growing cells of R2 on fungal spores/hyphae. Similarly, the antifungal activity of APB of R2 was evaluated by supplementing YME broth (20 mL) with 0.1, 0.2 and 0.4 mg of APB respectively, followed by inoculation with spores of *A. alternata* (10⁴ spores/mL). The YME broth without APB and inoculated with spores of *A. alternata* served as negative control, whereas the YME broth supplemented with methanol and inoculated with spores of *A. alternata* served as positive control. The flasks were incubated at 30 °C, 50 rpm in orbital shaker (Scigenics, India) for 48 h. The viability of *A. alternata* in flasks supplemented with different concentrations of APB was checked by transferring 10 µL of sample from each flask to the centre of YME agar plates. The plates were incubated at 30 °C for seven days and observed daily for the appearance of hyphal growth.

Further, 400 µL sample from each flask was centrifuged for 10 min at 10,000 rpm. The pellet was washed twice with sterile water and fixed on the surface of slide with 4% (v/v) glutaraldehyde for 12 h at 4 °C followed by washing with 0.1M sodium cacodylate buffer to remove excess of glutaraldehyde. This was followed by dehydration of fixed sample on slide with series of ethanol concentrations from 30% to 100% (v/v) and analyzed by using SEM-EVO LS-10 (Carl Zeiss, Germany).

Control of black point disease of wheat grains by APB of R2

The wheat grains were surface-sterilized by dipping them in 70% (v/v) ethanol for 5 min followed by 1 min exposure to 1% (v/v) sodium hypochlorite in distilled water and finally rinsed five times with sterile distilled water. Thirty grains were transferred to each flask (100 mL Erlenmeyer flasks) designated A, B and C. The grains in flasks A and B were inoculated with 100 µL spore suspension of *A. alternata* (10⁴ spores/mL), followed by addition of 1 mL of APB (50 mg/mL) to flask A and 1 mL of sterile water to flask B (positive control). The grains in flask C received only 1.1 mL of sterile distilled water (negative control). The flasks were shaken properly so as to allow even absorption of respective suspension by the grains. The flasks were incubated at 30 °C for seven days and grains in respective flasks were observed daily for appearance of disease related symptoms. After

7 days of incubation, 5 grains from each flask were sown in 15-well (1 well: 3 × 4 cm) seedling trays with 1 grain per well of tray. After 14 days, the shoot length, root length and dry weight (oven dried at 70 °C for three days) of plantlets were determined to evaluate the effect of different treatments on grains.

Statistical analysis

Data were subjected to one way analysis of variance (ANOVA) and the means were compared using Tukey's HSD at a significance level of $p \leq 0.05$ using AS-SISTAT.

Results

Isolation and screening

The results presented in Table 1 show the observations of initial screening in which six bacterial isolates supported best antifungal potential against different fungal pathogens. The highest among all the bacterial isolates screened was isolate R2, which supported more than 70% inhibition of all the tested fungi, except *R. oryzae* for which 56% inhibition was observed. In light of this broad spectrum activity the strain R2, isolated from rhizosphere of wheat, was selected for further studies.

Identification of isolate R2

The colonies of R2 on YME agar plates appeared off-white, round, wrinkled and mucoid. The cells are gram-positive, endospore former, rod-shaped with rounded ends. The cells were motile as observed under the microscope by hanging drop method. The isolate was positive for catalase and Voges-Proskauer (VP)

test and negative for methyl red (MR) test. The isolate tested positive for starch and casein hydrolysis and negative for lipid hydrolysis. These morphological and biochemical properties are typical features reported for genus *Bacillus* (Cappuccino & Sherman, 1999). The comparison of partial sequence of R2 gene coding for 16S rRNA with already known sequences in GenBank database identified the strain as *Bacillus vallismortis*, as it showed 100% similarity with strain DSM11031 (Fig. 1). The nucleotide sequence obtained was submitted to GenBank, under accession no. KC283030.

Analysis of acid-precipitated biomolecules (APB) by TLC and HPLC

The acid precipitation of biomolecules in cell free supernatant of R2 yielded 0.9 g/L of APB. The APB resolved into five spots on thin layer chromatogram with Rf value of 0.33-(I), 0.43-(II), 0.55-(III), 0.693-(IV) and 0.877-(V) (Fig. 2). These spots appeared white when the plates were sprayed with distilled water, indicating to all being different lipopeptides. The Rf values of biomolecules IV and V corresponded to standards of iturin A and surfactin (Sigma, USA) respectively. The HPLC analysis of APB preparation showed four peaks with different retention time (Rt) (Fig. 3). The peak with Rt 2.813 min matched with standard of iturin A (Rt 2.867), whereas peaks with Rt 3.077 min and 3.48 min matched with standard of surfactin (which has three peaks, each corresponding to different isoform of surfactin). An additional peak at 6.87 min was also observed which might be representing biomolecules I to III.

TLC agar overlay assay

It is evident from the bioautographs presented in Figs. 4a-g that for different fungal strains the level of

Table 1. Antifungal activity of the six best isolates against seven phytopathogenic fungi by dual culture plate assay

Fungal phytopathogens	Reduction in fungal growth (%)					
	R2	R5	R11	M32	RC16	M56
<i>Alternaria alternata</i>	85 ± 0.50 ^a	79 ± 1.52 ^a	80 ± 2.30 ^a	80 ± 1.15 ^{ab}	73 ± 1.00 ^{cd}	76 ± 1.52 ^b
<i>Fusarium oxysporum</i>	80 ± 0.50 ^c	78 ± 1.52 ^a	75 ± 1.00 ^{ab}	79 ± 1.15 ^{abc}	78 ± 1.15 ^{abc}	76 ± 2.08 ^b
<i>Fusarium moniliforme</i>	87 ± 1.10 ^{ab}	80 ± 2.08 ^a	70 ± 1.15 ^b	85 ± 1.52 ^a	80 ± 0.57 ^{ab}	84 ± 1.15 ^a
<i>Colletotrichum</i> sp	83 ± 1.70 ^b	78 ± 1.15 ^a	73 ± 1.52 ^b	78 ± 1.73 ^{abc}	82 ± 0.00 ^a	78 ± 1.15 ^{ab}
<i>Helminthosporium</i> sp	78 ± 1.10 ^d	75 ± 0.57 ^{ab}	74 ± 1.73 ^{ab}	76 ± 1.52 ^{bc}	75 ± 1.52 ^{bc}	77 ± 1.15 ^{ab}
<i>Rhizoctonia oryzae</i>	56 ± 1.70 ^f	43 ± 1.73 ^c	47 ± 1.00 ^c	34 ± 2.08 ^d	50 ± 1.52 ^e	36 ± 1.52 ^d
<i>Magnaporthe grisea</i>	73 ± 1.70 ^e	70 ± 1.52 ^b	72 ± 0.00 ^b	72 ± 2.00 ^c	68 ± 1.73 ^d	62 ± 1.52 ^c

*Values are the means of triplicate measurements ± standard errors; means within a column followed by the same letter are not significantly different, $p \leq 0.05$; based on Tukey's test.

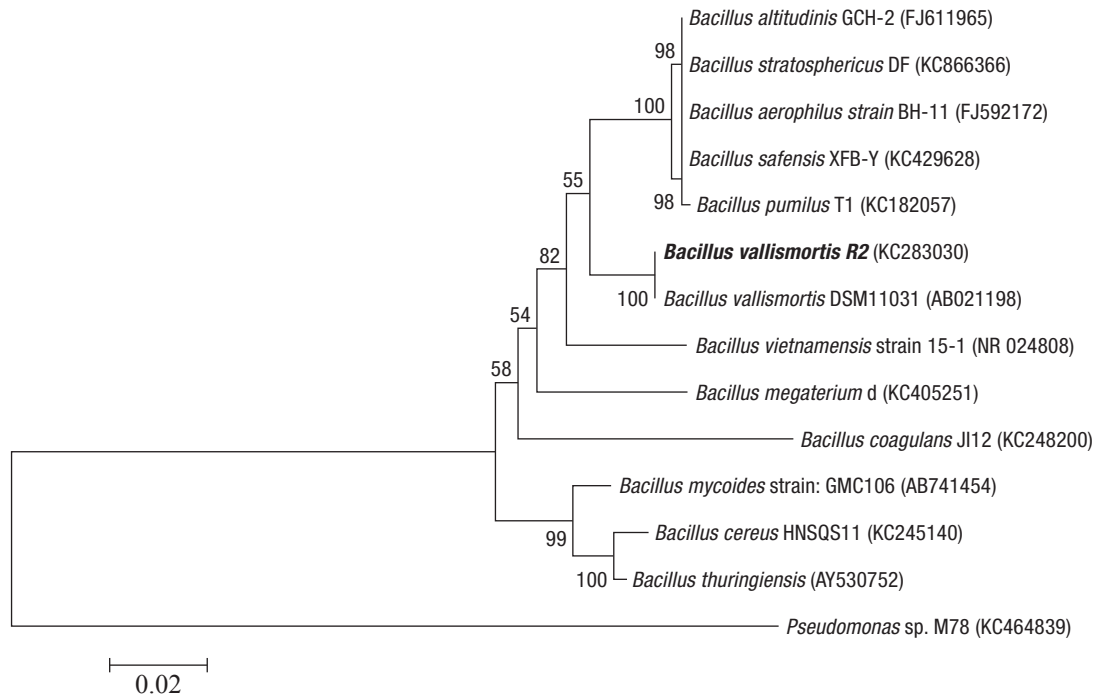


Figure 1. Phylogenetic analysis based on 16S rRNA gene sequences of isolate R2 constructed by using Neighbor-Joining method. The percentage of replicate trees with associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances (0.02) computed using the Kimura 2-parameter method is in the units of the number of base substitutions per site. The 16S rDNA accession numbers are given within brackets. Evolutionary analyses were conducted in MEGA5.

inhibition and appearance of the inhibition zones varied. The spots I to IV supported antifungal activity against all the fungi tested, whereas no antifungal activity was observed for surfactin like biomolecules at spot V.

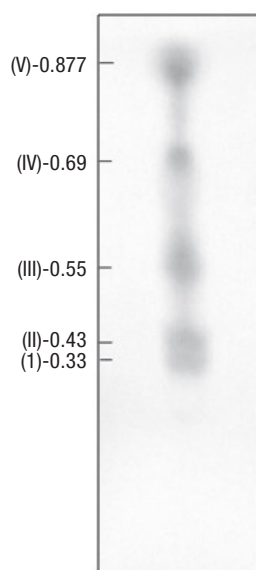


Figure 2. Thin layer chromatogram of resolved components of acid-precipitated biomolecules (APB) of isolate R2 exposed to iodine vapours.

A clear and overlapping inhibition zone was supported by spots I, II and III for *A. alternata*, *Helminthosporium* sp. and *F. moniliforme* along with signs of early sporulation in hyphae immediately around the inhibition zone (Figs. 4a-c). However, in *Colletotricum* sp and *M. grisea* a hazy inhibition zone was observed corresponding to these biomolecules (Figs. 4f-g). On the other hand, in the case of *F. oxysporum* and *R. oryzae*, distinct and overlapping inhibition zones were observed corresponding to spots I and II and a hazy zone of inhibition was evident at spot III (Figs. 4d-e). The iturin-like biomolecules at spot IV afforded a hazy inhibition zone against *A. alternata*, *F. moniliforme*, *Helminthosporium* sp, *F. oxysporum* and no inhibition zone against *R. oryzae*. However, a clear zone of inhibition was observed corresponding to spot IV for *Colletotricum* sp and *M. grisea*.

Microscopic evaluation of antifungal potential of R2 cells and their APB on *A. alternata* growth and morphology

Different morphological deformities viz. shrunken hyphal tips, disorganization of hyphae, swollen hyphae, excessive branching of hyphae and cell content degen-

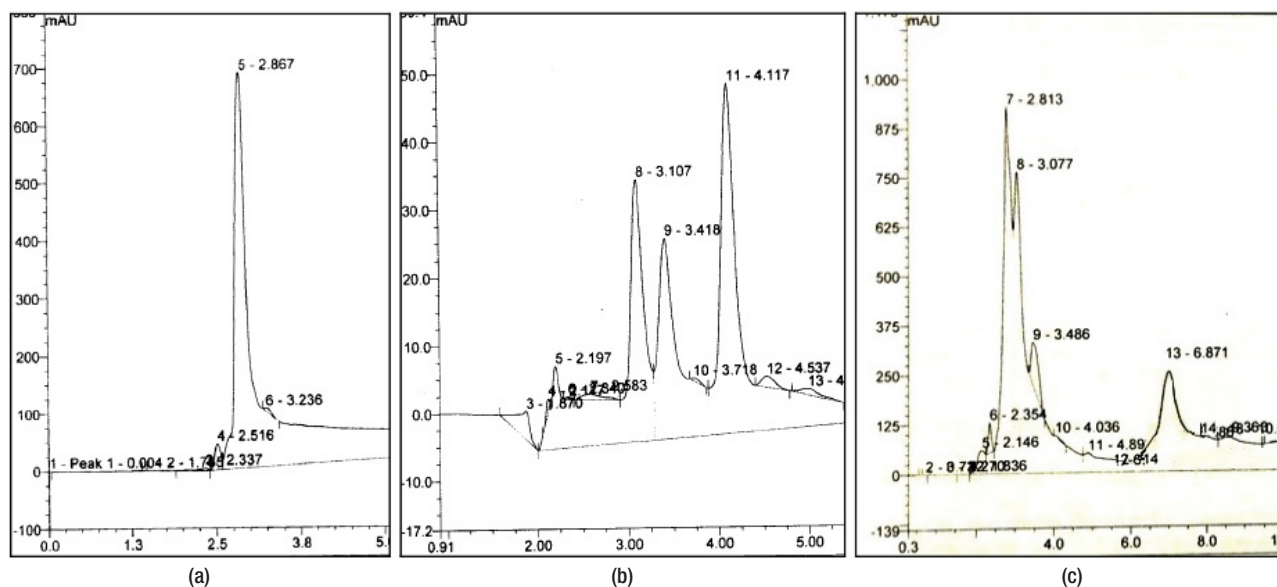


Figure 3. HPLC chromatograph of (a) standards of iturin, (b) standards of surfactin and (c) acid-precipitated biomolecules (APB) of R2.

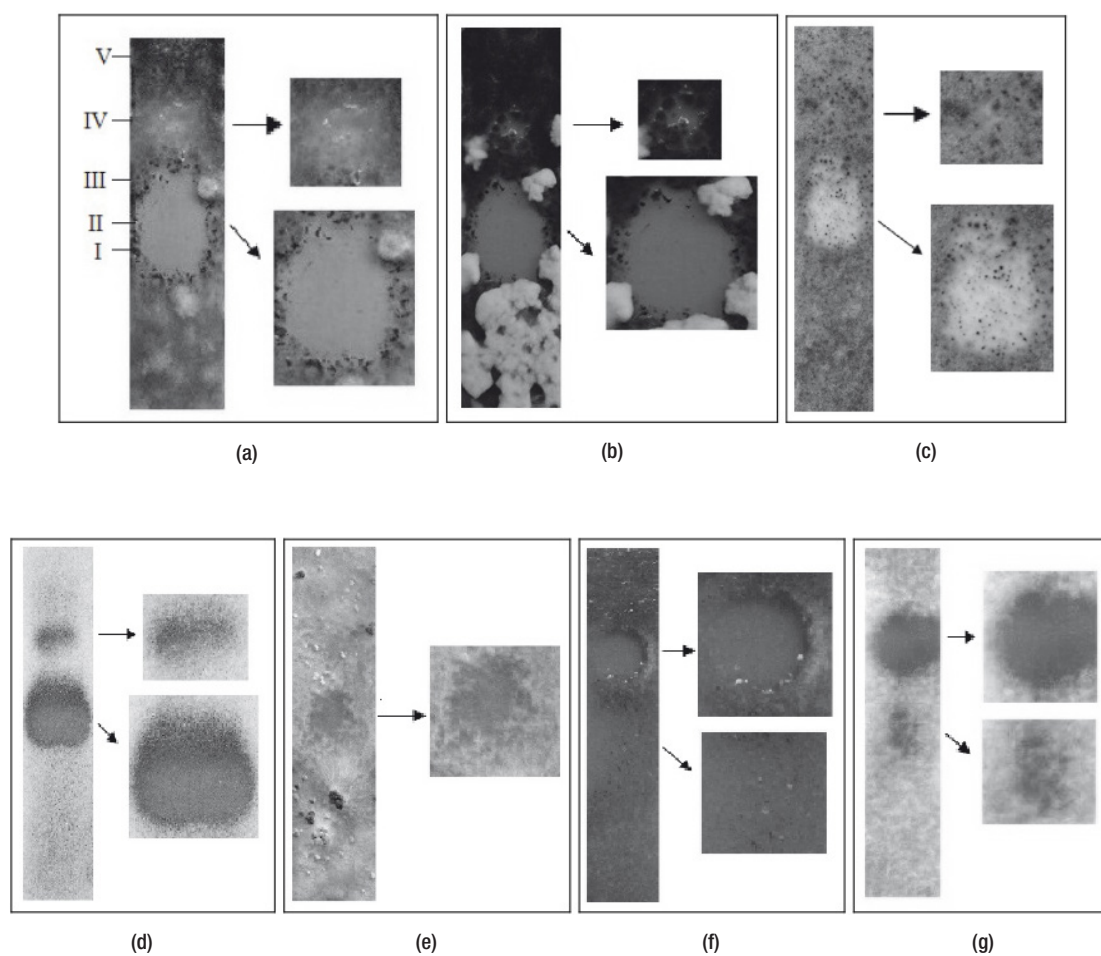


Figure 4. TLC agar overlay assay of resolved components of APB of R2 showing zones of inhibition against *A. alternata* (a), *Helminthosporium* sp (b), *F. moniliforme* (c), *F. oxysporum* (d), *R. oryzae* (e), *Colletotrichum* sp (f), and *M. grisea* (g).

eration were evident in plate assay where fungal hyphae were grown in presence of R2 cells as compared to control (Fig. 5). Scanning electron micrographs of fungal hyphae grown in presence of whole cells of R2 and different concentrations of APB showed shrunken, collapsed, empty hyphae, and large depressions and loss of turgidness in comparison to the regular morphological features in positive and negative control (Fig. 6).

The delay in fungal growth on YME agar plates after exposure to APB was directly proportional to increasing concentration of APB, as inoculum from flask treated with 0.1, 0.2 and 0.4 mg of APB showed mycelial growth on YME agar plate after 4, 5 and 7 days of incubation respectively, in comparison to samples taken from the positive and negative controls which showed mycelial growth after 2 days of incubation.

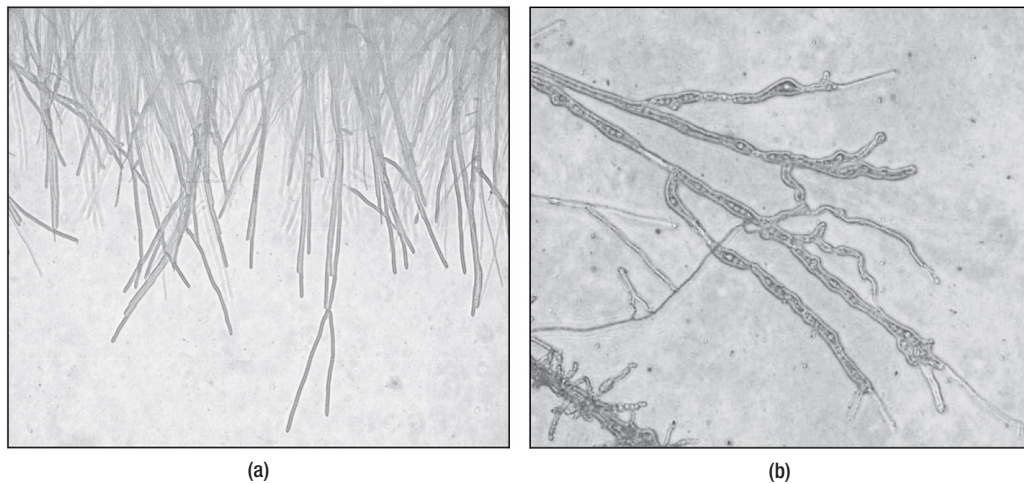


Figure 5. Antagonistic effect of growing cells of R2 on *A. alternata* by plate assay. Shrunk hyphal tips, swollen hyphae and excessive branching of hyphae (b), in comparison to control (a), in plates observed under inverted microscope.

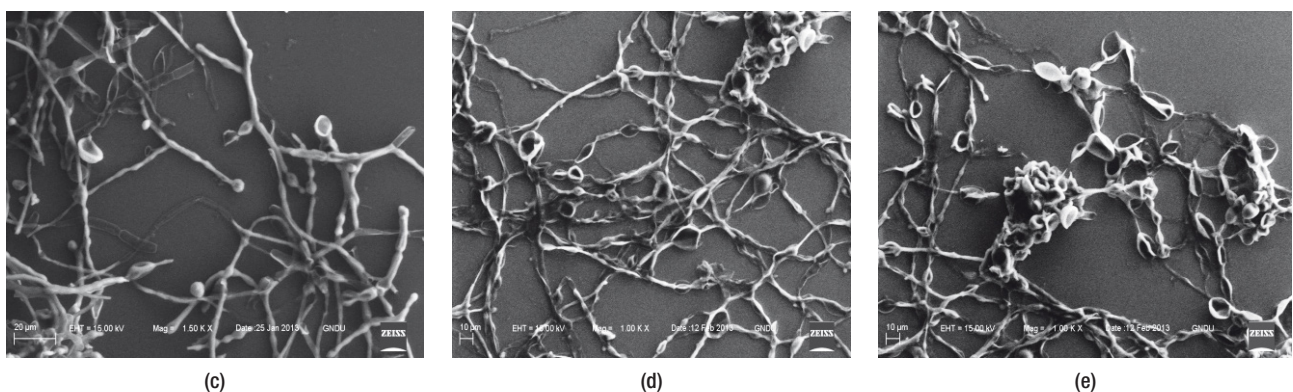
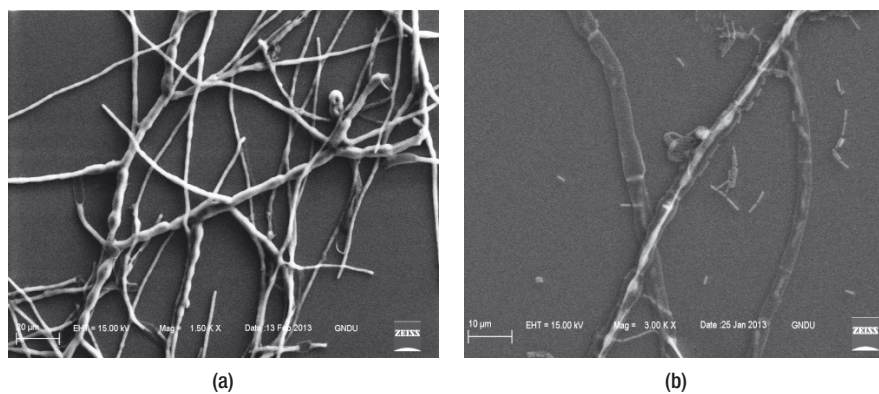


Figure 6. Scanning electron micrographs showing the growth of *A. alternata* (a) in absence of cells and APB of R2 (control); (b) in presence of cells of R2; and in medium supplemented with (c) 0.1 mg, (d) 0.2 mg and (e) 0.4 mg of APB of R2.

Control of black point disease of wheat grains by APB of R2

The ability of APB to inhibit spore germination of *A. alternata* was evidenced as the wheat grains in flask A treated with APB and inoculated with fungal spores were healthy, resisted development of the disease and appeared similar to grains in flask C which were neither exposed to fungal spores nor to APB (Fig. 7). Further, these grains germinated into healthy plants with no significant variation in their shoot length, root length and dry weight as compared to plantlets of control grains (Table 2). However, flask B grains inoculated with fungal spores and not treated with APB showed disease symptoms after 2 days of incubation as evidenced by the characteristic discolouration starting from the embryonic end. The whole grains turned black after 4 days of incubation due to fungal growth and when sown in soil did not germinate.

Discussion

The rhizospheric soil is known to support higher number of bacteria due to release of root exudates and the resulting interactions viz. symbiotic, parasitic, antagonistic etc. among the microbial population improve the chances of isolation of potential biocontrol agents (Whipps, 2001; Lugtenberg *et al.*, 2002; Villaciers *et al.*, 2003; Singh *et al.*, 2004). Different bacterial strains isolated from rhizospheric soil samples of wheat, rice, onion and tomato plants were screened for their antifungal potential against common phytopathogenic fungi prevalent in Punjab. The isolate R2, identified as *B. vallismortis*, achieving more than 50% inhibition of all the fungal strains in dual culture plate assay, was used for further studies. The APB of R2 resolved into five spots (I to V) as observed by exposing the TLC chromatogram to iodine vapours. The TLC and HPLC analysis of APB further indicated the presence of iturin A (IV) and surfactin (V) like compounds as compared

Table 2. Germination of wheat grains into plants, after infection and treatment with APB of isolate R2 in comparison to those in control

Treatments [†]	Shoot length (cm)	Root length (cm)	Dry weight (g)
Flask C	17.00 ± 0.94 ^a	10.00 ± 1.20 ^a	0.08 ± 0.00 ^a
Flask A	14.80 ± 1.15 ^a	10.75 ± 0.81 ^a	0.08 ± 0.00 ^a
Flask B	0.36 ± 0.22 ^b	0.10 ± 0.10 ^b	0.01 ± 0.00 ^b

Mean values (± standard error) of five plants followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey's test. [†]Flask C, uninfected wheat grains treated with sterile water, negative control; Flask A, wheat grains infected with *A. alternata* and treated with 1 mL APB of R2; Flask B, sterile water, positive control.

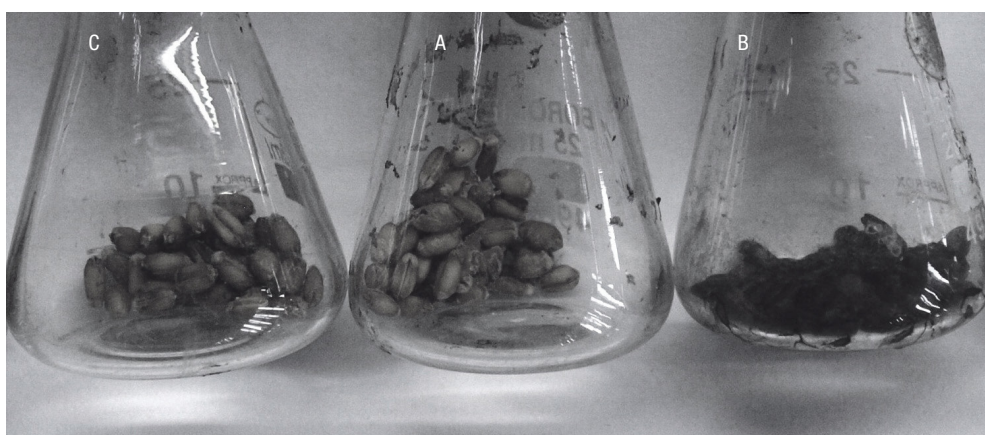


Figure 7. Antifungal potential of APB of R2 in controlling black point disease of wheat. Flask C, uninfected wheat grains treated with sterile water, negative control; Flask A, wheat grains infected with *A. alternata* and treated with 1 mL APB of R2; Flask B, sterile water, positive control.

to standard molecules. The spots I, II and III might be isoforms of fengycins as their R_f values on TLC and HPLC profile (R_t 6.87 min) were similar to those earlier reported in literature (Yanez-Mendizabel *et al.*, 2012; Grover *et al.*, 2010). These amphiphilic cyclic lipopeptide (CLP) compounds have been reported for their wide spectrum antifungal activity due to their interaction with biological membranes (Magnet-Dana & Peypoux, 1994; Stein, 2005). The TLC-based bioautography studies indicated varied antifungal potential of these biomolecules with the respective fungal pathogen. The clear zone may be due to the inhibition of spore germination and/or inhibition of hyphal growth by antifungal biomolecules. The hazy zones indicated an intermediate inhibition and induction of early sporulation due to activation of stress response in presence of APB. These variations in inhibition patterns may be attributed to interaction of lipopeptides with sterol molecules in cell membrane, which vary in different groups of fungi (Hu *et al.*, 2007). This was further confirmed by light and scanning electron microscopy, as Fig. 6b shows detachment of cell membrane from cell wall of fungal hyphae due to leakage of cytoplasmic fluid from the pores formed in the membrane by amphiphilic cyclic lipopeptides. Severity of deformities caused was directly proportional to the concentration of antifungal biomolecules in both agar plates and shake flask studies. The deformities observed were similar to those reported earlier, caused due to formation of pores by interaction of biomolecules with the fungal cell membrane resulting in shrunken and empty hyphae (Romero *et al.*, 2007; Makovitzki *et al.*, 2007). The formation of pores further causes an increased Ca²⁺ influx which has been reported to induce excessive branching of hyphae (Thrane *et al.*, 1999; Mortel *et al.*, 2009). The appearance of bulbous morphology may also be attributed to the effect of APB on the cytoskeletal elements, actin and tubulin leading to collapsed cytoskeleton and un-polarized growth at the tip of hyphae (Tendulkar *et al.*, 2007). The other cellular responses observed in bioautographs viz. inhibition of mycelial growth and early sporulation around the inhibition zone may be attributed to the induction and cross interaction between different signalling pathways viz “two component” histidine kinase, HOG1-mitogen activated protein kinase and SKN7-mediated ROS detoxification in fungal strains, which play an important role in regulating sporulation and other metabolic events in fungi (Kuang-Ren Chung, 2012).

In the State of Punjab, the incidence of black point disease is frequent, causing significant loss of wheat production. The incidence of this disease has also been reported in other parts of the world, *i.e.* Australia, North America and Europe with *A. alternata* and *A.*

infectoria as major species associated with this disease among the other *Alternaria* spp. (Perello *et al.*, 2008). In light of this, the potential of APB to control spread of black point disease in wheat grains was explored. The wheat grains treated with APB of R2 resisted development of disease when challenged with spores of *A. alternata* and retained their ability to grow into healthy plants as compared to wheat grains in positive control which failed to germinate due to severe damage inflicted by the fungal pathogen. The biomolecules of endophytic isolate *B. vallismortis* strain ZZ185, have been reported to control disease symptoms of root rot in wheat seedlings caused by *A. alternata* and *Fusarium graminearum*. The active biomolecules belonged to iturin family and were identified as Bacillomycin D (n-C14 and iso-C15) (Zhao *et al.*, 2010). Similarly, the biocontrol potential of *B. vallismortis* strain EXTN-1 against *Phytophthora capsici* and *Pectobacterium carotovorum* has been related to presence of seven iturin A analogs out of which only two analogs showed activity against fungal pathogens (Park *et al.*, 2014). As per reported literature and to the best of our knowledge, this is the first report related to evaluation of the ability of biomolecules of indigenous *B. vallismortis* to control the black point disease of wheat grains.

These first time observations indicated the presence of at least three different types of cyclic lipopeptides (CLPs) similar to surfactin, iturin and fengycins from isolate *B. vallismortis*. As per earlier reports mentioned above, only a CLP belonging to the family iturin has been identified from *B. vallismortis*. Further studies with purified molecules could provide insights into their synergistic interactions for improved antifungal potential. These basic studies could pave way for their future use as biocontrol agents along with other conventional chemical fungicides against fungal pathogens causing economic losses to diverse crops like wheat, rice, tomato, onion, sugarcane etc.

Acknowledgment

We acknowledge Dr. Yubee Gill, Associate Professor of the Department of English, Guru Nanak Dev University (Amritsar), for proof reading the manuscript.

References

- Cakmakci R, Erat M, Erdagan U, Figen-Donmez M, 2007. The influence of plant-growth-promoting rhizobacteria on growth and enzyme activities in wheat and spinach plants. J Plant Nutr Soil Sci 170: 288-295. <http://dx.doi.org/10.1002/jpln.200625105>

- Cappuccino GJ, Sherman N, 1999. Microbiology: A laboratory manual, 4th ed. Addison Wesley Publ Co, CA, USA.
- Dexter JE, Matsuo RR, 1982. Effect of smudge and black-point, mildewed kernels and ergot on durum wheat quality. *Cereal Chem* 59: 63-69.
- El-Ghaouth A, 1997. A biologically-based alternatives to synthetic fungicides for the control of postharvest diseases. *J Ind Microbiol Biotechnol* 19: 160-162. <http://dx.doi.org/10.1038/sj.jim.2900428>
- Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GA, 2008. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl Environ Microbiol* 74: 2461-2470. <http://dx.doi.org/10.1128/AEM.02272-07>
- Grover M, Nain L, Singh SB, Saxena AK, 2010. Molecular and biochemical approaches for characterization of antifungal trait of a potent biocontrol agent *Bacillus subtilis* RP24. *Curr Microbiol* 60: 99-106. <http://dx.doi.org/10.1007/s00284-009-9508-6>
- Hiltner L, 1904. Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderer Berücksichtigung der Gründüngung und Brache. *Arb DLG* 98: 59-78.
- Hu LB, Shi ZQ, Zhang T, Yang ZM, 2007. Fengycin antibiotics isolated from B-FS01 culture inhibit the growth of *Fusarium moniliforme* Sheldon ATCC38932. *FEMS Microbiol Lett* 272: 91-98. <http://dx.doi.org/10.1111/j.1574-6968.2007.00743.x>
- Kaur R, Macleod J, Foley W, Nayudu M, 2006. Gluconic acid: An antifungal agent produced by *Pseudomonas* species in biological control of take-all. *Phytochemistry* 67: 595-604. <http://dx.doi.org/10.1016/j.phytochem.2005.12.011>
- Kuang-Ren Chung, 2012. Stress response and pathogenicity of the necrotrophic fungal pathogen *Alternaria alternata*. *Scientifica* 2012: 1-17. <http://dx.doi.org/10.6064/2012/635431>
- Li L, Mo M, Qu Q, Luo H, Zhang K, 2007. Compounds inhibitory to nematophagous fungi produced by *Bacillus* sp. strain H6 isolated from fungistatic soil. *Eur J Plant Pathol* 117: 329-340. <http://dx.doi.org/10.1007/s10658-007-9101-4>
- Lugtenberg BJJ, Chin-A-Woeng TFC, Bloemberg GV, 2002. Microbe-plant interactions: principles and mechanisms. *A Van Leeuw J Microb* 81: 373-383.
- Magnet-Dana R, Peypoux F, 1994. Iturins, a special class of pore-forming lipopeptides: biological and physiological properties. *Toxicology* 87: 151-174. [http://dx.doi.org/10.1016/0300-483X\(94\)90159-7](http://dx.doi.org/10.1016/0300-483X(94)90159-7)
- Makovitzki A, Viterbo A, Brotman Y, Chet I, Shai Y, 2007. Inhibition of fungal and bacterial plant pathogens in vitro and in planta with ultrashort cationic lipopeptides. *Appl Environ Microbiol* 73: 6629-6636. <http://dx.doi.org/10.1128/AEM.01334-07>
- Mew T, 1991. Disease management in rice. In: *CRC Handbook of pest management in agriculture*, Vol. 3, CRC Press, Boston. pp: 279-299.
- Monaco C, Sisterna M, Perello A, Bello GD, 2004. Preliminary studies on biological control of the blackpoint complex of wheat in Argentina. *World J Microbiol Biotechnol* 20: 285-290. <http://dx.doi.org/10.1023/B:WIBI.0000023835.05701.10>
- Mortel JE, Tran H, Govers F, Raaijmakers JM, 2009. Cellular responses of the late blight pathogen *Phytophthora infestans* to cyclic lipopeptide surfactants and their dependence on G proteins. *Appl Environ Microbiol* 75: 4950-4957. <http://dx.doi.org/10.1128/AEM.00241-09>
- Park K, Ryu H, Dutta S, Lee SH, Lee SW, Moon SS, 2014. Identification of 7 different iturin compounds from *Bacillus vallismortis* inducing systematic resistance against *Phytophthora capsici* and *Pectobacterium carotovorum*. XVI Int Cong on Molecular Plant-Microbe Interaction (MPMI), Rhodes, Greece, July 6-10: P144.
- Perello A, Moreno M, Sisterna M, 2008. *Alternaria* infectoria species-group associated with black point of wheat in Argentina. *Plant Pathol J* 57: 379. <http://dx.doi.org/10.1111/j.1365-3059.2007.01713.x>
- Roberts P, Muchovej R, Achor D, Baker CA, Bruton B, Adkins S, 2005. Investigation into a mature watermelon vine decline and fruit rot. (Abstr.) *J Phytopathol* 95 (suppl): S89.
- Romero D, Vicente A, Olmos JL, Davila JC, Perez-Garcia A, 2007. Effect of lipopeptides of antagonistic strains of *Bacillus subtilis* on the morphology and ultrastructure of the cucurbit fungal pathogen *Podosphaera fusca*. *J Appl Microbiol* 103: 969-976. <http://dx.doi.org/10.1111/j.1365-2672.2007.03323.x>
- Singh DP, Goel LB, Kumar J, Sinha VC, Singh RK, Sharma I, Aujla SS, Singh A, Singh KP, Tiwari AN, 2003. Post-harvest surveys of wheat grains for the presence of Karnal bunt and black point disease in different agroclimatic zones of India. *Indian J Agr Res* 37: 264-268.
- Singh BK, Millard P, Whiteley AS, Murrell JC, 2004. Unravelling rhizosphere-microbial interactions: opportunities and limitations. *Trends Microbiol* 12: 386-393. <http://dx.doi.org/10.1016/j.tim.2004.06.008>
- Sisterna M, Sarandon S, 2000. Blackpoint incidence on durum wheat in Argentina: influence of cultivar and location. *Acta Agron Hung* 48: 395-401. <http://dx.doi.org/10.1556/AAgr.48.2000.4.9>
- Stein T, 2005. *Bacillus subtilis* antibiotics: structures, synthesis and specific functions. *Mol Microbiol* 56: 845-857. <http://dx.doi.org/10.1111/j.1365-2958.2005.04587.x>
- Szczeczek M, Shoda M, 2006. The effect of mode of application of *Bacillus subtilis* RB14-C on its efficacy as a biocontrol agent against *Rhizoctonia solani*. *J Phytopathol* 154: 370-377. <http://dx.doi.org/10.1111/j.1439-0434.2006.01107.x>
- Tendulkar SR, Saikumari YK, Patel V, Raghotama S, Munshi TK, Balram P, Chattoo BB, 2007. Isolation, purification and characterization of an antifungal molecule produced by *Bacillus licheniformis* BC98, and its effect on phytopathogen *Magnaporthe grisea*. *J Appl Microbiol* 103: 2331-2339. <http://dx.doi.org/10.1111/j.1365-2672.2007.03501.x>
- Thrane C, Olsson S, Nielsen TH, Sorensen J, 1999. Vital fluorescent stains for detection of stress in *Pythium ultimum* and *Rhizoctonia solani* challenged with viscosinamide from *Pseudomonas fluorescens* DR54. *FEMS Microbiol Ecol* 30: 11-23. <http://dx.doi.org/10.1111/j.1574-6941.1999.tb00631.x>
- Villacieros M, Power B, Sanchez-Contreras M, Loret J, Oruezabal RI, Martin M, Fernandez-Pinaz F, Bonilla I, Whelan C, Dowling DN, Rivilla R, 2003. Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meloti* in the

- alfalfa (*Medicago sativa*) rhizosphere. *Plant Soil* 251: 47-54. <http://dx.doi.org/10.1023/A:1022943708794>
- Whipps JM, 2001. Microbial interactions and biocontrol in the rhizosphere. *J Exp Bot* 52: 487-511. http://dx.doi.org/10.1093/jexbot/52.suppl_1.487
- Yanez-Mendizabal V, Zerrouh H, Vinas I, Torres R, Usall J, Vicente A, Perez-Garcia A, Teixido N, 2012. Biological control of peach brown rot (*Monilinia* spp.) by *Bacillus subtilis* CPA-8 is based on production of fengycin-like lipopeptides. *Eur J Plant Pathol* 132: 609-619. <http://dx.doi.org/10.1007/s10658-011-9905-0>
- Zhao Z, Wang Q, Wang K, Brian K, Liu C, Gu Y, 2010. Study of the antifungal activity of *Bacillus vallismortis* ZZ185 in vitro and identification of its antifungal components. *Biore-source Technol* 101: 292-297. <http://dx.doi.org/10.1016/j.biortech.2009.07.071>