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Full Length Research Paper

Molecular and biochemical characterization of surfactin producing *Bacillus* species antagonistic to *Colletotrichum falcatum* Went causing sugarcane red rot

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Bacillus **species suppress phytopathogens by producing lipopeptide antibiotics, hydrolytic enzymes, siderophores and other secondary metabolites. Three bacterial strains *Bacillus subtilis* NH-100 (EU627167), *B. subtilis* NH-160 (EU627169) and *Bacillus* sp. NH-217 (EU627170) with proven ability to suppress red rot disease on sugarcane plants were further characterized to elucidate the multiple modes of action involved in their biocontrol activity. Plate assays pointed out the production of protease and antibiotics. Lipopeptide antibiotic surfactin was detected in the culture extract of *B. subtilis* NH-160 and *Bacillus* sp. NH-217 through LC-MS (Liquid chromatography - mass spectrometry). These results were further supported by identifying the presence of *sfp* and *srfAC* genes of surfactin biosynthetic operon using specific polymerase chain reaction (PCR) primers. Two strains *B. subtilis* NH-160 and *Bacillus* sp. NH-217 were further analyzed for their survival in compost which successfully retained consistency in their population 4.0 - 5.0 log $CFUg^{-1}$ after 14th day. Bacteria capable of suppressing pathogens and maintaining their population by competing with other microbes can be successfully utilized as biopesticide for sustainable organic farming.**

Key words: *Bacillus*, biocontrol, surfactin, sugarcane, red rot.

INTRODUCTION

The excessive use of chemical pesticides has caused soil pollution and detrimental effects on human beings. Accordingly, the use of eco-friendly biocontrol agents of plant pathogens has been greatly accentuated (Correa et al., 2009). These agents suppress pathogens by different mode of actions (Liu et al., 2009).

Bacillus species are outstanding biocontrol agents with proven excellent characteristics like effective root colonization, versatile activity against multiple pathogens and promising ability to sporulate (Kloepper et al., 2004; Romero et al., 2004). This assures their ubiquitous occurrence in the environment and use in the framework of integrated disease management (Correa et al., 2009). Cyclic lipopeptides of the surfactin, iturin and fengycin families are one of the important metabolites produced by *Bacillus* species and their involvement in disease control have been widely reported (Ongena and Jacques 2008). They impart successful biocontrol activity by direct suppression of phytopathogens and reinforcing of the potential host plant through stimulating induced systemic resistance phenomenon. These lipopeptides are the products of multimodular enzyme complexes called non-ribosomal peptide synthetases (NRPs). These molecules are usually synthesized as isoforms with variation in the fatty acid chain length and peptide part. Surfactin, exhibiting strong antibiotic activity, consists of heptapeptides containing a -hydroxy fatty acid with 13 to

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15 carbon atoms. Genes involved in surfactin biosynthesis are encoded by the *srf* operon which consist of four open reading frames (ORFs) designated as *srf*A-A, *srf*A-B, *srf*A-C and *srf*A-D (Peypoux et al., 1999; Yakimov et al., 1998). These open reading frames contain seven modules organized in a linear array. Similarly, fengycin biosynthesis is encoded by an operon containing five ORFs viz *fen*A, *fen*B, *fen*C, *fen*D and *fen*E while the ORFs *itu*A, *itu*B, *itu*C and *itu*D are involved in iturin biosynthesis (Steller et al., 1999). In our previous work, we have reported that three *Bacillus* strains isolated in our laboratory suppressed red rot disease caused by *Colletotrichum falcatum* on sugarcane plants, and also showed good abilities to colonize sugarcane roots (Hassan et al., 2010) . The aims of this study were to detect the production of various antifungal metabolites produced by these promising biocontrol agents to elucidate the underlying mechanisms responsible for their antagonistic activity towards *C. falcatum* and evaluate their survival in compost to formulate these agents as biopesticide.

MATERIALS AND METHODS

Bacillus strains and growth conditions

The antagonistic strains *Bacillus subtilis* NH-100 (EU627167), *B. subtilis* NH-160 (EU627169), *Bacillus* sp. NH- 217 (EU627170) and red rot pathogen *C. falcatum* were isolated from the sugarcane plant and rhizospheric soil (Hassan et al., 2010). Isolates were grown at 37°C on Luria Bertani (LB) agar routinely and preserved in 20% glycerol at -80°C for a long time. *C. falcatum* was cultured on potato dextrose agar (PDA).

Production of extracellular metabolites, HCN, protease and antibiotics

Production of extracellular metabolites was determined as described by Montealegre et al. (2003) with certain modifications. The antagonistic isolates were grown in sterile nutrient broth (NB) for 6 days on a rotary shaker at 175 rpm at 28±2°C. Cell free supernatant of these strains obtained by centrifugation was mixed with PDA (Oxoid chemicals) at the rate of 5, 15 and 25% (v/v). LB instead of cell free supernatant was added in control PDA plates. A 5 mm mycelial disk of *C. falcatum* was kept at the centre of petri dish and incubated at 28±2°C. Production of diffusible and volatile antibiotics was determined as described by Montealegre et al. (2003). Mycelial growth of the fungus was observed after 5 - 6 days and percentage inhibition was determined using the following formula:

% Inhibition = [1 - (Fungal growth/control growth)] × 100

Production of protease and HCN was tested according to the method of Denizci et al. (2004) and Sun et al. (2006) respectively.

LC-MS analysis

The presence of lipopeptide antibiotics such as surfactin, fengycin and iturin etc in the supernatants of *B. subtilis* strain NH-160 and *Bacillus* sp. NH-217 were determined by liquid chromatography mass spectrometry (LC-MS) analysis. Bacterial strains were grown in Landy medium and incubated at 28 ±2°C for 96 h in an orbital shaker at 250 rpm. Cell free supernatant was obtained by centrifugation at 13000 rpm for 10 min passing through 2 µm syringe filter. The supernatants were extracted with methanol (Ahimou et al., 2000) while methanolic fractions were analyzed by LCT (Liquid Chromatograph Time of Flight Mass Spectrometer with Electrospray sample introduction; Waters Incorp).

Detection of genes involved in antibiotic synthesis

Genomic DNA was isolated from the *Bacillus* strains by standard protocols (Maniatis et al., 1982). Primers used for detecting genes involved in lipopeptide antibiotics (surfactin, fengycin, iturin) synthesis are described in Table 1. New primers were designed by retrieving the reported sequences from GenBank (http://www.ncbi.nih.gov) and aligning at clustal W (www.clustalw). PCR amplifications were carried out in 50- L reaction mixtures containing PCR buffer (Qiagen Inc.), 1.5 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Qiagen Inc.), 40 g of each forward and reverse primer, 200 M each of dATP, dGTP, dCTP, and dTTP and 2 L of template DNA (approximately 100 ng of bacterial genomic DNA). The amplifications were performed using a thermocycler (Eppendorf) with the cycle conditions mentioned in Table 1. Amplified products were separated by electrophoresing on 1.2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. Desired products were eluted from gels using the gel extraction kit (Qiagen Inc) and sequenced either directly or by cloning in TOPO T/A cloning vector (*Invitrogen*) on the sequencer ABI at the Central Genomics and Sequencing facility, University of Sheffield, UK. Nucleotide sequences were identified using the basic local alignment search tool (BLAST) and GenBank nucleotide data bank from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih. gov/).

Survival of antagonistic *Bacillus* strains in compost

Development of soil microcosms

The microcosm was developed by filling the 50-mL falcon tubes with 20 g non-sterilized soil-based compost obtained from the Department of Animal and Plant Sciences, University of Sheffield, UK. The falcon tubes were placed at temperature of 23±2°C.

Introduction of antagonistic strains to compost

Single colonies of *B. subtilis* NH-160 and *B.* Sp. NH-217 were cultured in LB for 24 h at 30°C on an orbital shaker at 200 rpm. The cells (10^9 CFU m/L) were mixed with 25% Ringer solution (2.25 g/LNaCl, 0.15 g/l KCl, 0.12 g/l $CaCl_2$, 0.05 g/l Na_2CO_3). The washed cells were mixed with compost in each falcon tube with a concentration of 10^8 CFU/g. The control treatment received ringer solution only without cells.

Enumeration of antagonistic Bacillus

The compost was sampled periodically on day 0th, 1st, 2nd, 4th, 7th, 14th and 21st after bacterial inoculation and *Bacillus* species were isolated according to the method of Hart et al. (1998). The inoculated strains were identified on the basis of their colony morphology, antagonistic activity and the presence of molecular marker that is, *sfp* gene.

Table 1. The primers used in this study and PCR profile.

Primer	Sequence	Antibiotics	Product size/ Amplification	PCR profile	Reference
Sfp-f	ATG AAG ATT TAC GGA ATT TA	Surfactin	675/Yes	95°C - 5 min. 30 cycles (94°C for 1 min, 48°C for	Hsieh et al. (2004)
Sfp-r	TTATAAAAGCTCTTCGTACG			1 min, 72°C for 1 min) and 72°C -5 min.	
SrfA-f	GAT CAG GTT CAR GAY ATG TAT TA	Surfactin	3700/Yes	95°C - 5 min. 30 cycles (94°C for 1 min, 55°C for	This study
SrfA-r	AGC ATT TCT GCG TGY GTK CC			3 min,72°C for 3 min) and 72°C -15 min.	
Fend-f	TCC TGC AGA AGG AGA AGT GA	Fengycin	281/No	95°C - 5 min. 30 cycles (94°C for 1 min, 54°C for	This study
Fend-r	CGT CTT CCG TTT CTA AAA TGG T			1 min,72°C for 1 min) and 72°C -5 min.	
BmD-f	AAT CTT GCC TTT TTA TTT CCK G	Iturin	1200/No	95°C - 5 min. 30 cycles (94°C for 1 min, 48°C for	This study
BmD-r	TTA TTT TAA AAT CCG CAA TTS TTC C			1 min,72°C for 1 min) and 72°C -5 min.	

* Degeneracy code: S= C or G, Y= A or T, K= G, T.

Table 2. Biocontrol characteristics of *Bacillus* strains isolated from sugarcane rhizosphere.

Strains	Inhibition of *C. falcatum* (%)					Survival in soil based compost							Production of antifungal metabolites				
	*Extracellular metabolites			**Antibiotics													
	5%	15%	25%	Dif	Volt	0th	1st	2nd	4th	7th	14th	21st	HCN	Prt	srf	Itr	Fen
B. subtilis NH-160	12.3 ^{b}	24.3 ^{b}	40 ^{b}	43 ^{b}	15.2 ^{b}	7.7 ^{a}	7.8 ^{a}	7.6 ^{a}	6.5 ^{a}	5.6 ^{a}	5 ^{a}	5 ^{a}	--	++	+	-	-
Bacillus sp. NH-217	18 ^{a}	32 ^{a}	48 ^{a}	52 ^{a}	27.5 ^{a}	7.5 ^{a}	7.5 ^{a}	7.1 ^{a}	5.5 ^{b}	4.1 ^{b}	4.2 ^{a}	4.2 ^{a}	--	--	+	-	-
B. subtilis NH-100	10 ^{b}	25 ^{b}	41 ^{b}	39 ^{b}	13.5 ^{c}	ND	ND	ND	ND	ND	ND	ND	--	++	+	-	-
Control	0 ^{c}	0 ^{c}	0 ^{c}	0 ^{c}	0 ^{d}	ND	ND	ND	ND	ND	ND	ND	++	+++	-	-	-
P.flourescence CHA0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-	-	-

HCN; SRF; ITR; FEN = + production; -- no production; PRT: ++ = zone = 5 mm; +++ = zone > 5 mm. Values are mean of three replicates and those bearing same letter are significantly same according to Duncan' s multiple range test at $p < 0.05$.
*LSD $_{0.05}$ (5%) = 4; LSD $_{0.05}$ (15%) = 3.9; LSD $_{0.05}$ (25%) = 1.9 ** LSD $_{0.05}$ (dif) = 7 and LSD $_{0.05}$ (vol) = 2.1.

Statistical analysis

All the data were subjected to analysis of variance (ANOVA) using computer statistical package MSTAT-C. Data values of bacterial colonies were log-transformed before analysis.

RESULTS

Detection of biocontrol determinants

Extracellular metabolites, diffusible and volatile antibiotics, HCN and hydrolytic enzymes are the major determinants of biocontrol activity. Only 2 out of 4 strains produced protease while all the strains were negative for HCN production (Table 2). The strain *Bacillus* sp. NH-217 showed maximum inhibition of fungus mycelium up to 53% by producing diffusible antibiotics and 28% through volatile antibiotic production followed by the strain *B. subtilis* NH-160 which caused 43 and 15% inhibition by producing diffusible and volatile antibiotics respectively. Inhibition of fungus by extracellular metabolites and antibiotics of the strains is shown in Table 2.

Detection of lipopeptide antibiotics produced by *Bacillus* strains

LC-MS analysis (Figure 1) of the crude lipopeptide extracts of strains *B. subtilis* NH-160 and *Bacillus* sp. NH-217 yielded a parent peak at m/z 542 along with the intense fragments at m/z 549 indicating the presence of surfactin antibiotic as detected by using KEGG (Kyoto Encyclopedia of

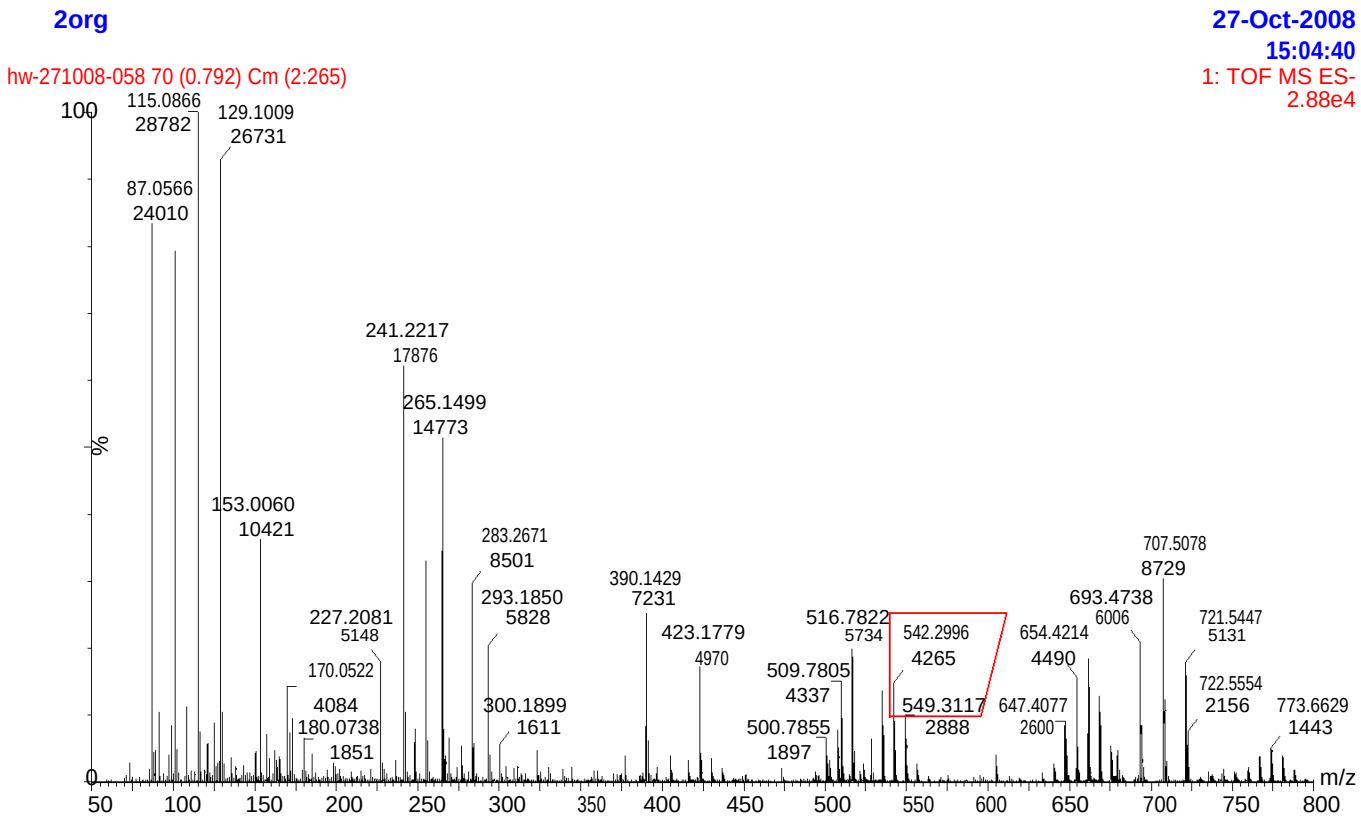

Figure 1. LC-MS analysis of the lipopeptide antibiotics produced by *Bacillus subtilis* NH-160. Peak at m/z 542 along with the intense fragments at m/z 549 indicating the presence of surfactin antibiotic as detected by using KEGG pathway database.

Genes and Genomes) pathway database.

Amplification of antibiotic related genes

Four primer pairs were used for the amplification of genes involved in the antibiotics biosynthesis from the *Bacillus* strains in this study. Primers *sfp*-f and *sfp*-r amplified a 629 bp of the *sfp* gene (Figure 2a) encoding 4'-phosphopantetheine transferase involved in surfactin biosynthesis from the *B. subtilis* NH-100, *B. subtilis* NH-160 and *Bacillus* sp. NH-217. A 3700 bp of *srf* AC gene (Figure 2b) involved in surfactin biosynthesis were also amplified from the *B. subtilis* NH-100 and *B. subtilis* NH-160 by using primers *srfA*-f, *srfA*-r. There was no amplification for *fenD* and *BmD* genes related to fengycin and Iturin antibiotic. Accession numbers of the partial nucleotide sequences of *sfp* and *srf* AC obtained from GenBank are FJ711067, FJ711068, FJ711069, FJ711070 and FJ711071.

Survival of *Bacillus* strains in un-sterilized compost

Population of *B. subtilis* NH-160 decreased until 14th day from 7.7 - 5 (log CFU/g) and then remained constant even at the 21st.day (Table 2) while *Bacillus* sp. NH -217 showed a decreased trend in the number of colonies from 7.5 - 4.1 (log CFU g^{-1}) during the initial seven days which afterwards persisted in this population level until the 21st day (Table 2).

DISCUSSION

Biocontrol activity of *Bacillus* strains against multiple plant pathogens have been widely reported and well documented (Correa et al., 2009; Kloepper et al., 2004). Their success as biocontrol agent is associated with the prominent property of producing lipopeptide antibiotics which exhibit wide spectrum antifungal activity, regulate attachment of microbes with various surfaces and enhance survival in the habitat (Sun et al., 2006). In our present study, the cell free supernatants of three *Bacillus* strains have effectively inhibited the mycelial growth of *C. falcatum*. Percentage inhibition of red rot pathogen was similar to the inhibition level of other pathogens by antagonistic *Bacillus* strains reported previously but our experiments were based on supernatants and sugarcane red rot pathogen rather than vegetative cells and other

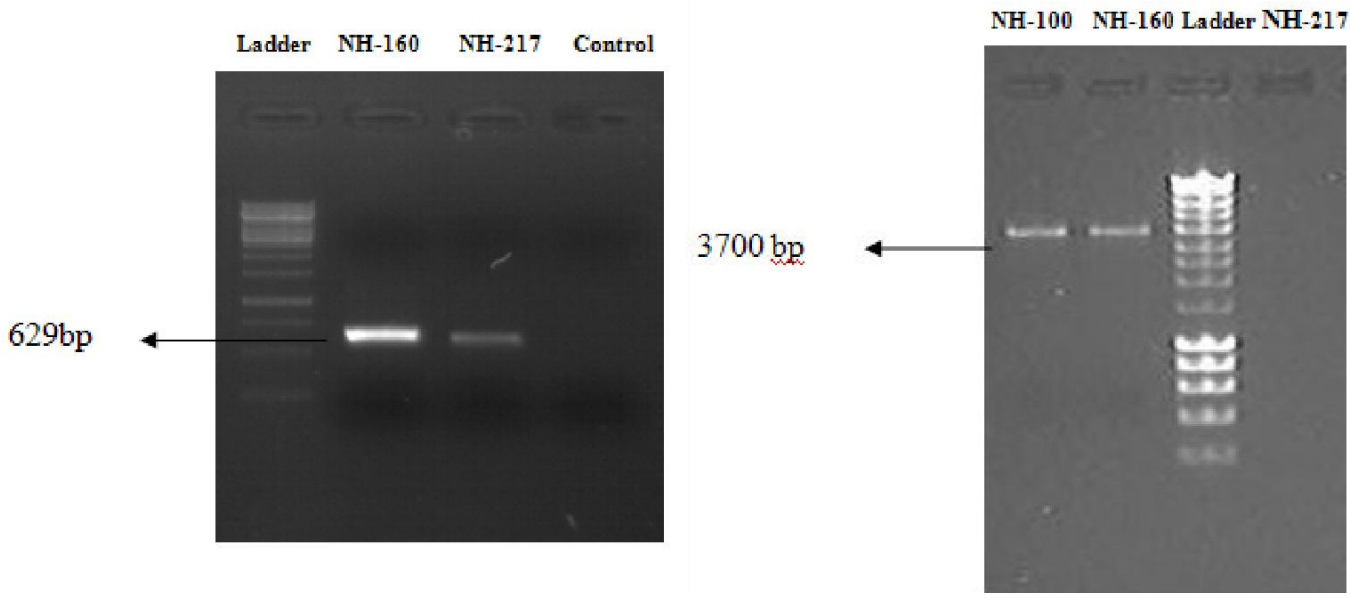

Figure 2. Amplification of (a) 629 bp *sfp* gene involved in surfactin synthesis (b) 3700 bp *srf*A gene involved in surfactin synthesis.

pathogenic fungi (Romero et al., 2004). Further plate assays indicated production of protease, diffusible and volatile antibiotics.

However, production of siderophores could not be assessed by plate assay due to inability of these strains to grow on the media described by Schwyn and Neilands., (1987). These findings supported the agreement with previous reports that antagonistic bacteria suppress pathogens by producing hydrolytic enzymes and antibiotics (Chen et al., 2008; Liu et al., 2009). Inability of strains to produce HCN will make them biocontrol agents of choice as HCN imposes negative effects on plant growth (Schippers et al., 1990). The LC-MS analysis of cell free supernatant attributed the presence of only surfactin- a lipopeptide antibiotic with proven antifungal activity against many pathogens (Yakimov et al., 1998). The *Bacillus* strains exhibiting pronounced antagonistic activity on sugarcane plants *in vivo* (Hassan et al., 2010) were found to be surfactin producer. These findings make the strains *B. subtilis* NH-100, *B. subtilis* NH- 160 and *Bacillus* sp. NH-217 valuable and rare as most reported strains are co-producers of iturin and fengycin along with surfactin. Moreover, co-production of these lipopeptides results in purification problems. As surfactin is an industrially important compound, so a strain producing only surfactin would be really ideal.

Genes encoding surfactin synthesis are common to numerous antagonistic *Bacillus* sp used as commercial biopesticide and strains carrying such genes possess a pronounced capability to suppress the soil-borne pathogens (Joshi and Gardener, 2006). Comprehensive genetic analysis of vegetative cells also proved the occurrence of *sfp/ srfAC* genes and absence of *fend*, *bmd genes* involved in surfactin, fengycin and iturin synthesis respectively in *B. subtilis* NH-100, *B. subtilis* NH-160 and *Bacillus* sp. NH-217.

The coherence of results by chemical and genetic analysis has further strengthened the authenticity of LC-MS analysis coupled with identification of compounds using KEGG pathway (Hashimoto et al., 2006). Survival of the two antagonistic strains *B. subtilis* NH-160 and *Bacillus* sp. NH -217 was investigated in soil based compost for 21 days. Population was monitored by culture dependent assay by taking advantage of sporulation ability of these strains which were easily cultured on media after exposing the sample at high temperature. The inoculated strains were further confirmed by detecting *sfp* gene as a molecular marker. Both strains showed good survival until the 21st day. Population of strain NH-160 decreased from day 0 to day 14 but it remained constant from day 14 to day 21, while the population dynamics of NH-217 decreased over day 7 and remained constant afterwards (Figure 2). The stability in survival of *Bacillus* strains may be attributed to their ability to sporulate and produce surfactin; a characteristic which supports their persistence under extreme conditions (Kloepper et al., 2004; Romero et al., 2004). Initial decline in population may be due to competition with the micro flora present in un-sterilized compost. This indicates the strong competitive ability of surfactin producing strains *B. subtilis* NH-160 and *Bacillus* sp. NH-217 which has also been proved previously in root colonization experiments (Hassan et al., 2010). Moreover, it can be predicted that these strains will maintain a high level of population in sterilized formulations.

Results suggest that the strains *B. subtilis* NH-100, *B. subtilis* NH-160 and *Bacillus* sp. NH-217 are unique in their characteristics like being antagonistic to *C. falcatum,* deficient in HCN production and producer of only surfactin lipopeptide. Hence, these strains can be valuable candidates in context to develop biopesticide for sugarcane red rot. However, further studies are required to formulate these strains in suitable carrier material and explore their potential under field conditions.

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