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Characterization of Antifungal Antibiotic Synthesis Genes from Different Strains of *Bacillus subtilis*

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In this study, fifteen bacterial strains were collected from different microhabitats and characterized using biochemical and molecular techniques. The use of 16S rRNA revealed that the strains were belonged to *Bacillus subtilis*. Further, the strains were evaluated for their efficacy to promote plant growth in tomato seedlings. The results showed the greater plant growth promotion in seedlings treated with *B. subtilis* strain EPCO16. Subsequently, they were evaluated against *Fusarium oxysporum* f. sp. *lycopersici in vitro*. *Bacillus subtilis* strain EPCO16 has greater inhibition to *F. oxysporum* f. sp. *lycopersici.* The PCR amplification of antibiotic genes in different strains of *B. subtilis* was carried out. The results revealed the presence of Iturin D, Iturin C, Bacillomycin A, Bacillomycin D, Bacilysin, Fengycin and β-glucanase in *B. subtilis* EPCO16. The characterization of antibiotic genes revealed the biocontrol potential of *B. subtilis* strain EPCO16 against soil borne pathogens of vegetables.

Key words: *Bacillus subtilis*, 16S rRNA, Lipopeptides, β-glucanase.

Tomato (*Solanum lycopersicum* Mill.) is a vital vegetable produce cultivated all over the world. Tomato cultivation is frequently hindered by vascular wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* (Synder and Hans). Sporulating Gram positive bacteria like *Bacillus spp*. have been used effectively for plant disease control and its role as biocontrol agent for managing the pathogen in crop plants are well reported (Hanene *et al.*, 2012). Many *Bacillus* species are proficient of producing a extensive range of secondary metabolites that are diverse in structure and function. *Bacillus subtilis* harbored a diversity of non-ribosomally formed small lipopeptides belonging to the Surfactin family: Surfactin and Lichenysins (Kluge *et al*., 1988); the Iturin family: Iturin A, C, D, and E, Bacillomycin D, F, and L and the Fengycin family: Fengicins and Plipastatins; as well as aminopolyols such as Zwittermycin A. Detection of antibiotic production in a particular bacterium is important in determining its competence to be a good biocontrol agent for plant diseases. Screening candidate strains for particular antibiotic-encoding sequences followed by direct detection of the antibiotic profile of a particular bacterium provides a rapid approach in comparison with the conventional method of selection (de Souza and Raaijmakers, 2003). PCR based detection of antibiotic biosynthetic genes is very appropriate approach in this regard. The current exploration was to study the identification of different antibiotics produced by *Bacillus subtilis* collected from different microhabitats and analyzing its efficient control against tomato wilt pathogen.

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MATERIALS AND METHODS

Fungal pathogen and Pathogenicity

Diseased tissues from collar and root regions of tomato cultivar (PKM 1) showing wilt symptoms were collected from a farmer's field near Devarayapuram, Coimbatore, Tamil Nadu. The pathogen was isolated in PDA. The Pathogenicity was proved and used for further studies.

Biocontrol agents

Potential bioantagonistic bacteria were isolated from Rhizosphere soil of healthy tomato plants from different locations of Tamil Nadu, India. The bacterial antagonist *Bacillus* spp. was isolated by serial dilution technique using nutrient agar media. The bacterial antagonists (TBR1, TBR2, TBR3, TBR4, TBR5, COMB3, COMB4, COMB5, COGB27, COGB29, COKPB25 and COPB16) were further purified on their respective media and compared with the strains maintained in laboratory. Bacterial endophytes *Bacillus subtilis viz.*, EPCO16, EPC5, and EPC8 were obtained from the Culture Collection Section, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Seed bacterization and Growth promotion

The endophytic and rhizosphere bacteria were grown on NA broth with constant shaking at 150 rpm for 48h at room temperature (28 ±2°C). After growth, the bacterial suspension was centrifuged and the precipitate was then resuspended in phosphate buffer. The concentration was adjusted using a spectrophotometer to approximately 10^8 cfu/ml (OD595=0.3) and used as bacterial inoculum (Thompson, 1996). Tomato seeds (cv.PKM 1) were surface sterilized with 2% sodium hypochlorite for 30 sec, rinsed in sterile distilled water and dried overnight under sterile air stream. Bacterial strains grown on NA broth were taken in a conical flask. Hundred gram of seeds were soaked in bacterial suspension containing 3×10^8 for 2 h and dried under shade. Plant growth promoting activity of bacterial strains was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Three replications were maintained for each treatment. The vigour index was calculated by using the formula as described by Baki and Anderson, 1973.

Vigour index = Percent Germination×seedling length

In vitro* efficacy of *B. subtilis* against *Fusarium oxysporum* f. sp. *lycopersici

The *B. subtilis* strains were evaluated against soil borne fungi, *Fusarium oxysoporum* f. sp. *lycopersici* by dual culture technique as described by Morton and Strouble, 1955. Three replications were maintained for each treatment

Biochemical characterization of *Bacillus subtilis*

Characterization included growth at different temperatures and the ability to utilize different substrates as a sole carbon source. Other than this the bacterial biochemical response was tested by using ready biochemical kit for the specific identification of gram positive rods (Rapid biochemical identification test kits-KB002 HiAssortedTM Biochemical test, HiMedia laboratories Pvt. Ltd). Results of these tests were scored either as positive or negative and grouped with the aid of manual in the kit.

Extraction of *Bacillus* DNA

DNA was extracted from Endophytic and Rhizosphere *Bacillus* spp. grown in nutrient broth or on nutrient agar plates at 28°C using a commercially available DNA extraction protocol (GeneiPureTM Bacterial DNA Purification Kit), whereby the cells were resuspended in lysis solution with lysozyme and incubated at 37°C for 30 min, followed by addition of Proteinase K, 20% SDS containing 0.3% beta-mercaptoethanol. DNA was purified by organic extraction and ethanol precipitation. Purified DNA was quantified by UV spectrophotometry.

Polymerase Chain Reaction

To prove strains as *Bacillus* sp., 16S rRNA superseding sequence specific BCF1 (CGGGAGGCAGCAGTAGGGAAT); BCR2 (CTCCCCAGGCGGAGTGCTTAAT) primers were used to get an amplicon size of 546 bp. PCR reactions were carried out as per the protocol of Rajendran *et al.*, 2008 The PCR products were photographed using gel documentation system (Alpha Innotech Corporation, USA), sequenced and accession numbers were obtained.

PCR detection of antibiotic biosynthesis genes and Sequencing

Primers and all the reagents were obtained from Bangalore Genei Pvt. Ltd., Bangalore,India. The primers used for molecular analyses are given in Table 3. PCR amplifications were carried out in 20-µl reaction mixtures. Samples were quickly

transferred in Mastercycler gradient (Eppendorf, Germany). Thermal cycling conditions were referred by the corresponding authors given in Table 3. A total of 5µl of each amplification reaction was analyzed by electrophoresis using a 1.5% agarose gel followed by ethidium bromide staining and ultraviolet visualization. Sequencing of antibiotic biosynthetic genes of *B. subtilis* was done and accession numbers were retrieved from the GenBank database.

Statistical Analysis

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines Gomez and Gomez, 1984. The percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at significant level ($P < 0.05$) and means were compared by Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Biocontrol agents and efficacy

In this study, the endophytic strain EPCO16, identified as belonging to *B. subtilis*, found to increase the vigour index of tomato seedlings significantly when compared to untreated control. Bacterial strains namely EPCO16, EPC8, TBR2, TBR3, TBR4, TBR5, COMB1, COMB4, COGB29 and COKPB25 were found to increase the vigour index of tomato seedlings significantly when compared to untreated control (Table 1). The *Bacillus* strain EPCO16 (Endophytic cotton strain) showed high inhibition to *F.oxysporum* f.sp. *lycopersici* followed by COMB3 (coconut rhizosphere strain), COKPB25 (coconut rhizosphere strain) and TBR1 (Tomato rhizosphere strain). The percent inhibition was significantly higher in plates streaked with EPCO16 (46.04 %), COMB3 (29.54 %), COKPB25 (28.33 %) and TBR1 (15.00 %) against control plates (Table 1). Similar information was given by Podile and Laxmi, 2008 in Pigeonpea and effectively inhibited the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici in vitro*. The inhibition zone could be due to the effect of diffusible inhibitory substances produced by the bacteria, which suppressed the growth of *Fusarium oxysporum*. Indeed, according to Crawford *et al.* 1993, the presence and size of the zone of inhibition have been used as evidence of the production of antibiotics by the bacteria.

Biochemical characterization

In the study, EPCO16, EPC5 showed

Table 1. *In vitro* efficacy of *Bacillus subtilis* strains

Treatments	Germination (%)	*Vigour index	Percent Inhibition over control
EPCO 16	96.63^{a}(80.85)	2311.46^{a}	46.04^{a}(42.72)
EPC 5	94.31^{abc}(76.40)	1217.86^{f}	16.16^{h}(23.69)
EPC 8	92.43^{abc}(74.05)	1923.51^{b}	25.97^{e}(30.64)
COMB 1	96.00^{ab}(79.87)	1908.48^{bc}	22.66^{g} (28.42)
COMB 3	88.50^{cd}(70.24)	1627.24^{d}	15.00^{i}(22.78)
COMB 4	94.00^{abc}(76.30)	1861.48^{bc}	13.00^{k}(21.13)
TBR 1	96.32^{a}(79.60)	2281.02^{a}	29.54^{b}(32.91)
TBR 2	94.00^{abc}(76.28)	1573.84^{de}	14.88^{j}(22.68)
TBR 3	92.74^{abc}(74.62)	1762.06^{c}	25.97^{e}(30.64)
TBR 4	94.00^{abc}(76.43)	1923.52^{b}	27.22^{d}(31.44)
TBR 5	86.00^{d}(68.15)	1849.00^{bc}	12.33^{l}(20.55)
COGB 27	90.00^{bcd}(71.80)	1620.00^{d}	12.33^{l}(20.55)
COGB 29	96.00^{ab}(79.05)	1877.08^{bc}	14.88^{j}(22.68)
COKPB 25	80.00^{e}(63.47)	1898.40^{bc}	28.33^{c}(32.15)
COPB 16	80.00^{e}(63.45)	1480.24^{e}	23.11^{f}(28.73)
CONTROL	76.00^{e}(60.69)	1486.56^{e}	0.00^{m}

*Mean of three replications

In a column, mean followed by a common letter are not significantly different at the 5% level by DMRT.

Values in the parenthesis are arscine transformed values

Table 2. Biochemical characterization of *Bacillus* isolates collected from different ecosystems of Tamil Nadu

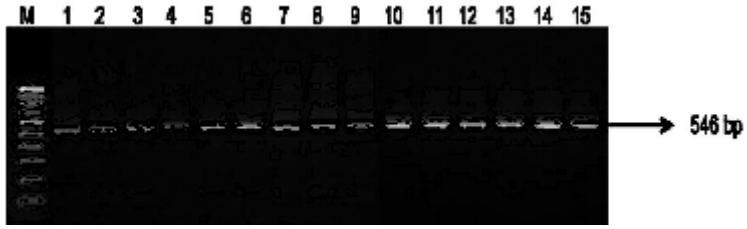
S. No	TEST	EPCO 16	EPC 5	EPC 8	COMB 1	COMB 3	COMB 4	TBR 1	TBR 2	TBR 3	TBR 4	TBR 5	COGB 27	COGB 29	COKPB 25	COPB 16	Control
1	Malonate	-	-	+++	-	-	-	-	-	+++	-	+++	-	-	-	-	-ve
2	Voges Proskauer's	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-ve
3	Citrate	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-ve
4	ONPG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-ve
5	Nitrate Reduction	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	——	+++	+++	+++	+++	-ve
6	Catalase	+++	+++	+++	+++	++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	-ve
7	Arginine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-ve
8	Sucrose	+++	+++	-	+++	+	+++	-	-	-	-	-	+	-	++	-	-ve
9	Mannitol	+++	++	-	++	+	+	-	-	-	-	-	+	-	+	-	-ve
10	Glucose	+++	+++	-	+++	-	+++	-	-	-	-	-	+	+	+++	-	-ve
11	Arabinose	+++	++	-	++	-	++	-	-	-	-	-	+	-	+	-	-ve
12	Trehalose	+++	+++	-	+	-	++	-	-	-	-	-	+	-	+	-	-ve
13	Gram staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
14	Endospore staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
15	KOH test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
16	Anaerobic growth	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
17	Growth at 45° C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve
18	Growth at 4° C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
19	Growth in 7% NaCl	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve
20	Siderophore production	+++	+++	+++	+++	++	+++	+++	++	+++	++	+++	+++	+++	++	+++	-ve
21	HCN production	++	+++	++	+	-	+	+	++	+	++	-	-	-	++	++	-ve
22	Volatile production	+++	+++	+++	++	++	-	++	-	-	-	-	-	-	-	-	-ve

+ve, positive reaction; +, Low production; ++, Medium production; +++, strong production; -ve,negative reaction; -, no production.

Table 3. PCR detection of antibiotic biosynthesis genes from *Bacillus subtilis*

Antibiotic Primer Type	Gene	Primer Name	Sequence (5' – 3')	Pcr Product Size/Detected	Authors	Accession Number
ITURIN D	*ituD, ituA*	ITUP4-F ITUP5-R	CCCCTGTTCTAGATGATCGGA GGAATCTCTGCATCGATTCTGTCCA TCTAACCGGCATC	800bp/yes	Tsuge *et al.*, 2001	JN257106* JN257107**
BACILLOMYCIN D	bamD	ITUD-F1 ITUD-R1	TTGAAYGTCAGYGCSCCTTT TGCGMAAATAATGGSGTCGT	482 bp/yes	Chung *et al*., 2008	JN257108*
BACILYSIN	bacD	BACAB-F1BACAB-R1	CTTCTCCAAGGGGTGAACAG TGTAGGTTTCACCGGCTTTC	815 bp/yes	Chung *et al*., 2008	JN226116 *
ITURIN C	ITU C	ITUC-F ITUC-R	TTCACTTTTGATCTGGCGAT CGTCCGGTACATTTTCAC	506 bp/yes	Baysal *et al.*, 2008	JN257110*
BACILLOMYCIN A	Bmy A	BACA-F BACA-R	TGAAACAAAGGCATATGCTC AAAAATGCATCTGCCGTTCC	344 bp/yes	Baysal *et al.*, 2008	JN257111* JN257112**
FENGYCIN D	Fen D	FEN D-F FEN D-R	CCTGCAGAAGGAGGAGAAGT GAAGTGCTCATCGTCTTCCGTTTC	220 bp/yes	Baysal *et al.*, 2008	JN257113*
MYCOSUBTILIN	fenF	ITUD-F1 ITUD-R1	TTGAAYGTCAGYGCSCCTTT TGCGMAAATAATGGSGTCGT	482 bp/yes	Chung *et al*., 2008	JN257109**
SURFACTIN	sfp	SFP-FSFP-R	ATG AAG ATT TAC GGA ATT TA TTATAA AAG CTC TTC GTA CG	675 bp/yes	Hsieh *et al*., 2004	HQ711611[a] HQ711610 [b]
β- GLUCANASE	encoding PR2 protein	β- GLU- F β-GLU- R	AATGGCGGTGTATTCCTTGACC GCGCGTAGTCACAGTCAAAGTT	400 bp/yes	Baysal *et al.*, 2008	JN257102[b], JN257103*, JN257104**, JN257105[a],

**B.subtilis* EPCO 16; ** *B.subtilis* TBR1 ; [a] *B.subtilis* EPC 8; [b] *B.subtilis* EPC 5



M. 100bp ladder
Lane 1 - EPCO16 (EF139864) Lane 2 – TBR1 (JN211013) Lane 3 – EPC5 (EF139862)
Lane 4 – EPC8 (EF139863) Lane 5 – TBR2 (JF918976) Lane 6 – TBR3
Lane 7 – TBR4 Lane 8 – TBR5 Lane 9 - COMB1
Lane 10 - COMB3 Lane 11 - COMB4 Lane 12 - COGB29
Lane 13 - COGB27 Lane 14 - COKPB25 Lane 15 - COPB16

Fig. 1. Detection of *Bacillus subtilis* by gene specific primer BCF1 and BCR2

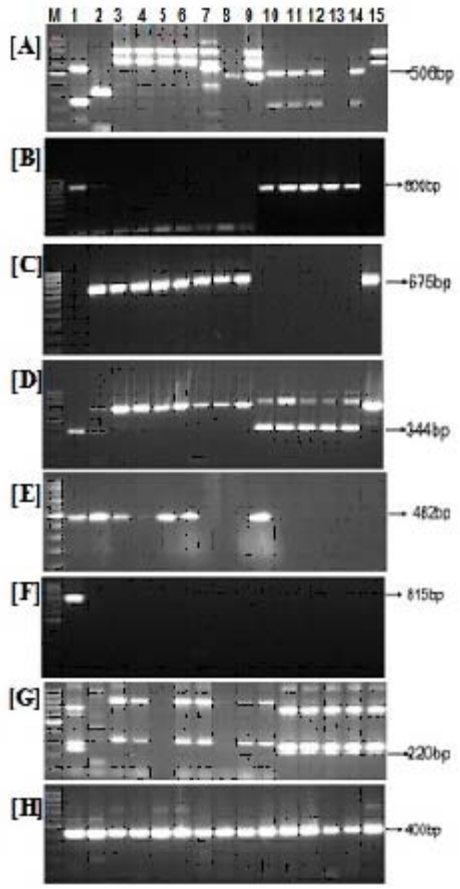


Fig. 2. PCR amplification of gene corresponding to Iturin C [A], Iturin D [B], Surfactin [C]. Bacillomycin A [D], Bacillomycin D [E], Bacilysin [F], Fengycin D [G], β-glucanase [H]. Lane M. 100bp ladder; Lane 1 - EPCO16; Lane 2 - TBR1; Lane 3 - EPC5; Lane 4 - EPC8; Lane 5 – TBR2; Lane 6 – TBR3; Lane 7 – TBR4; Lane 8 – TBR5; Lane 9 - COMB1; Lane 10 - COMB3; Lane 11 - COMB4; Lane 12 - COGB29; Lane 13 - COGB27; Lane 14 - COKPB25; Lane 15 - COPB16

positive for all the tests except Voges-Proskauer's, Malonate and ONPG tests. The results for other *Bacillus* strains were shown in Table 2.

Molecular characterization by PCR

The ITS primers amplified a fragment size of 546bp corresponding to the region of the 16s-23s rRNA intervening sequence for *Bacillus* sp. The results of PCR amplification has further confirmed that these strains were pertaining to the group of *Bacillus* (Fig. 1)

Detection of antibiotic genes through PCR

PCR was used to screen for genes involved in the production of antibiotics like Iturin C, Iturin D, Surfactin, Bacilysin, Bacillomycin A, Bacillomycin D, Fengycin, Mycosubtilin and β-glucanase by *Bacillus* strains (Table 3, Fig. 2). Analysis of partial sequence of the 800-bp PCR product of *Bacillus subtilis* EPCO16 from reactions with the ITUP4-F/ITUDP5-R primer pair showed 98% identity with the Iturin D biosynthesis gene cluster. Analysis of partial sequence of the 815-bp PCR product *Bacillus subtilis* EPCO16 strain using the BACAB-F1/BACAB-R1 primer pair showed 95% identity with bacilysin sythetase A (bacA), a gene involved in bacilysin biosynthesis. Similarly maximum similarity was obtained for other partial sequence of the PCR product of *Bacillus subtilis* strains. To the best of our knowledge, this study reports the production of seven antibiotics viz., Iturin D, Iturin C, Bacillomycin A, Bacillomycin D, Bacilysin, Fengycin and β-glucanase by a single *Bacillus subtilis* EPCO16 strain for the first time. This implies that Surfactin and Iturin are among the most common lipopeptide antibiotics produced by *Bacillus sp*. The specific surface and membrane-active properties of Surfactins assist the bacteria

to form biofilms; therefore, surfactin is thought to perform developmental functions rather than defense functions in the environment. This might be the reason that most *Bacillus* strains produce Surfactins. On the other hand, Surfactin and Iturin (Iturin A, Bacillomycin D, etc.) are found to exhibit strong antifungal activity (MagetDana and Peypoux, 1994) and Surfactin is considered the most powerful biosurfactant known, which causes detergent like action on biological membranes Carrillo *et al.,* 2003. Surfactin and members of the Iturin family, therefore, might have the ability to exert significant pressure on pathogens.

In the current PCR screening, all strains were positive for production of β-glucanase a gene encoding PR2 protein. A similar finding was made by Baysal *et al.,* 2008 in *Bacillus subtilis* strain (EU07) against *Fusarium oxysporum* f. sp. *radicis-lycopersici.* In addition, our present study detected the presence of antibiotic synthesis genes for Mycosubtilin in *Bacillus subtilis* TBR1 strain, which had not been detected in all 21 strains tested by Athukorala *et al.,* 2009 The detection of antibiotic synthesis genes and their production by the *Bacillus* strains in the current study revealed that one of the mechanisms responsible for biocontrol exhibited by these strains could be antibiosis. Among the strains tested, the contribution of antibiotics in biocontrol could be very high for strains EPCO16 and TBR1 in which several antibiotics were detected. Using *Bacillus subtilis* as a biocontrol agent, it has several advantages over other organisms because of its resistance to heat and desiccation (Hou *et al.,* 2006). By understanding the mechanisms of biocontrol at the molecular and biochemical level will enable us to develop coherent strategies for the application of the antagonists and their metabolites for agricultural purposes.

REFERENCES

1. Athukorala, S.N.P., Fernando, W.G.D., Rashid, K.Y. Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. *Can J Microbiol.,* 2009; **55**: 1021-1032.
2. Baki, A.A.A., Anderson, J.D. Vigour determination in soybean seed by multiple criteria. *Crop Sci.,* 1973; **31**: 630-633.
3. Baysal, O., Caliskan, M., Yesilova, O. An inhibitory effect of a new *Bacillus subtilis* strain (EU07) against *Fusarium oxysporum* f. sp. *radicis-lycopersici. Physiol. Mol. Plant P.,* 2008; **73**: 25–32.
4. Carrillo, C., Teruel, J.A., Aranda, F.J., Ortiz, A. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochimica et Biophysica Acta – Biomembranes.,* 2003; **161**: 91–97.
5. Chung, S., Kong, H., Buyer, J.S., Lakshman, D.K., Lydon, J., Kim, S.D., Roberts, D. Isolation and partial characterization of *Bacillus subtilis* ME488 for suppression of soilborne pathogen of cucumber and pepper. *Applied Microbial and Cell Physiology.,* 2008; **80**: 115–123.
6. Crawford, D.L., Lynch, J.M., Whips, J.M., Ousley, M.A. Isolation and characterization of *actinomycetes* antagonists of fungal root pathogen. *Appl. Environ. Microbiol*., 1993; **59**: 3899-3909.
7. de Souza, J.T., Raaijmakers, J.M. Polymorphisms within the prnD and pltC genes from pyrrolnitrin and pyoluteorin-producing *Pseudomonas* and *Burkholderia* spp. *FEMS Microbiol. Ecol*., 2003; **43**: 21–34.
8. Gomez, K.A., Gomez, A.A. Statistical Procedure for Agricultural Research. John Wiley and Sons, New York. 1984.
9. Hanene, R., Abdeljabbar, H., Marc, R., Abdellatif, B., Ferid, L., Najla, S. Biological control of Fusarium foot rot of wheat using fengycin-producing *Bacillus subtilis* isolated from salty soil. *Afr. J. Biotechnol*., 2012; **11**: 8464-8475.
10. Hou, X., Boyetchko, S.M., Brkic, M., Olson, D., Ross, A., Hegedus, D. Characterization of the anti-fungal activity of a *Bacillus* spp. associated with sclerotia from *Sclerotinia sclerotiorum*. *Appl. Microbiol. Biotechnol*., 2006; **72**: 644–653.
11. Hsieh, F.C., Li, M.C., Lin, T.C., Kao, S.S. Rapid Detection and Characterization of Surfactin-Producing *Bacillus subtilis* and Closely Related Species Based on PCR. *Current microbiol*., 2004; **49**: 186-191.
12. ISTA. Proceedings of the international Seed Testing Association, International Rules for Seed Testing. *Seed Sci. Technol.,* 1993; **21**: 25-30.
13. Kluge, B., Vater, J., Salnikow, J., Eckart, K. Studies on the biosynthesis of surfactin, a lipopeptide antibiotic from *Bacillus subtilis* ATCC 21332. *FEBS Lett*., 1988; **231**: 107-110.
14. MagetDana, R., Peypoux, F. Iturins, a special class of poreforming lipopeptides: biological and

physicochemical properties. *Toxicology.*, 1994; **87**: 151-174.

15. Morton, D.T., Stroube, W.H. Antagonistic and stimulatory effects of microorganism upon *Sclerotium rolfsii*. *Phytopathol.*, 1955; **45**: 419-420.
16. Podile, A.R., Laxmi, V.D.V. Seed Bacterization with *Bacillus subtilis* AF 1 Increases Phenylalanine Ammonia-lyase and Reduces the Incidence of Fusarial Wilt in Pigeonpea. *Phytopathol.*, 2008; **146**: 255–259.
17. Rajendran, L., Karthikeyan, G., Raguchander, T., Samiyappan, R. Cloning and Sequencing of Novel Endophytic *Bacillus subtilis* from Coconut for the Management of Basal Stem Rot Disease. *Asian J. Plant Pathol.*, 2008; **2**: 1-14.
18. Thompson, D.C. Evaluation of bacteria immunization; an alternative to pesticides for control of plant disease in greenhouse and field. *The Biological Control of Plant Disease.* 1996; 30-40.
19. Tsuge, K., Akiyama, T., Shoda, M. Cloning, sequencing, and characterization of the iturin A operon. *J. Bacteriol.*, 2001; **183**: 6265-6273.