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Exploiting Biocontrol Potential of *Bacillus amyloliquefaciens* (sic.) Fukumoto for the Management of Mungbean Anthracnose

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Experiments were conducted during 2017 to 2018 to manage anthracnose in mungbean caused by *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scrib using Bacillus spp. A total of eight *Bacillus* spp were evaluated of which *Bacillus amyloliquefaciens* (sic.) Fukumoto recorded significantly the highest growth inhibition of 54.8 per cent. The second best antagonist was *B. licheniformis* with *C. lindemuthianum* by 48.14 per cent and it was followed by *B. endophyticus* which inhibited mycelial growth by 41.11 per cent. *Bacillus* spp tested for the presence of lipopeptide biosynthetic genes *viz.*, Iturin A, Fengycin, and Surfactin, all the eight strains showed positive for fengycin and seven strains were positive for Iturin A and six strains were positive for surfactin. All the three antibiotic genes were found in *B. amyloliquefaciens*. The crude antibiotics were extracted from *B. amyloliquefaciens* associated with inhibition of *C. lindemuthianum*. The presence of antifungal compounds in secondary metabolities were identified by gas chromatography mass spectrophotometry (GC-MS). The strain *B. amyloliquefaciens* was formulated in liquid for pot culture studies. Seed treatment with *B. amyloliquefaciens* @ 10 ml/kg seed + foliar spray of *B. amyloliquefaciens* @ 10ml/ litre at 25 DAS was found to be effective in reducing the incidence of anthracnose in mungbean by 62.05 per cent. This treatment also recorded increased plant height, pods per plant and per plant yield.

Key words: Mungbean anthracnose, *Bacillus amyloliquefaciens*, Lipopeptide genes, GC-MS

Mungbean or green gram [*Vigna radiata* (L.) Wilczek], is an important ancient leguminous crop of India. It is a versatile crop grown for its seeds, green manure and forage and it is considered as “Golden Bean”.

In Tamil Nadu, mungbean is cultivated in an area of 2.29 lakh ha with the production of 1.87 lakh tonnes and a productivity of 787 kg ha^{-1}. India produced 1.50 mt of mungbean from an area of 3.02mha with the productivity of 498 kg ha^{-1}. Anthracnose causes considerable losses in a large number of crops *viz.,* cereals, coffee and legumes. The anthracnose is prevalent in all mungbean growing states of India. The anthracnose was reported to cause yield loss upto 100 per cent (Padder *et al*., 2017).

Many fungal and bacterial antagonists display remarkable antagonistic activity against wide range of pathogens. Among these beneficial bacteria, *Bacillus* spp considered as an excellent biocontrol agent due to their multiple mode of action *viz.,* myco parasitism, plant growth promotion (Correa *et al*., 2009), production of antibiotics, hydrolytic enzyme and induced systemic resistance (Dalal *et al.,* 2014). Stankovic *et al.,* (2012) tested 205 natural isolates of *Bacillus* spp for the presence of biosynthetic genes.

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Material and Methods

In vitro screening of Bacillus spp *against C. lindemuthianum*

The antifungal activity of eight Bacillus spp obtained from culture collection, Department of Plant Pathology, TNAU, Coimbatore were tested against *C. lindemuthianum* by dual culture technique using PDA medium. A mycelial disc (5mm) of the pathogen was placed at one end of the Petri plate. The bacterial antagonists were streaked one cm away from the periphery of the Petri plate just opposite to the mycelial disc of the pathogen. The plates inoculated with the pathogen alone served as the control. Three replication were maintained for each treatment. The plates were incubated at 28 ± 2°C for 15 d. After incubation the growth of the pathogen and inhibition zone was measured. The Percent inhibition (PI) over control was calculated as below.

$$PI = \frac{C-T}{C} \times 100$$

where, PI – Percent inhibition, C – Radial growth of pathogen in control, T – Radial growth of pathogen in treatment

List of Bacillus spp used for *in vitro screening against C. lindemuthianum*

Bacillus strains	Accession number

B. licheniformis	MG241257
B. altitudinus	MG241237
B. amyloliquefaciens	MG241252
B. endophyticus	MG241258
B. sonorensis	MG241231
B. aryabattai	MG241277
B subtilis Cab_5	KP412481.1
B subitilis Ccb_7	KR265028.1

Efficacy of Bacillus* spp *on plant growth promotion

The *Bacillus* strains were grown in nutrient broth with constant shaking at 150 rpm for 48 h at room temperature (28±2°C). The bacterial cells were harvested by centrifugation at 10,000 rpm for 15 min and re-suspended in phosphate buffer (0.01 M, pH 7.0). The concentration was adjusted using a spectrophotometer to approximately 108 cfu ml^{-1} (OD_{595}=0.3) and used as bacterial inocula.

Plant growth promoting activity of *Bacillus* isolates were assessed based on the seedling vigour index by the standard roll towel method as recommended by ISTA, 1999. Seeds of mungbean (SML 1082) were surface sterilized with two per cent sodium hypochlorite for 30 s, rinsed in sterile distilled water and dried overnight under sterile stream of air. The Bacillus suspension was taken in Petri plate and 100 mg of carboxymethylcellulose (CMC) was added as an adhesive material. One gram of mungbean seeds were soaked in 10 ml of bacterial suspension (containing $3x10^8$ cfu ml^{-1}) for 2 h and dried overnight in a sterile Petri plate. After soaking, the Seeds were placed in moistened germination paper slip over the seeds and gently pressed. The germination papers were rolled along with polythene sheets to prevent drying of the papers and incubated in growth chamber for seven days. Three replications were maintained for each treatment. Root length and shoot length of individual seedlings were measured and the germination per cent of seeds were recorded. The vigour index was calculated by the formula as described by Abdul-Baki and Anderson (1973).

Vigour Index = (Mean root length + Mean shoot length) x Germination per cent

***Molecular detection of lipopeptide antibiotic genes of Bacillus* spp**

The genomic DNA from all the eight isolates of *Bacillus* spp was isolated using the standard protocol of Cetyl Trimethyl Ammonium Bromide (CTAB) method proposed by Knapp and Chandlee, (1996) .

PCR amplification

Iturin A

The forward primer ITUD1F (5'GATGCGAT CTCCTTGGATGT3') and reverse primer ITUD1R (5'ATCGTCATGTGCTGCTTGAG3') were used for amplification of iturin A gene (647 bp) (Ramarathnam *et al.,* 2007). The 20µl PCR mixture contained approximately 50ηg of total DNA, 5 mM each dNTPs, 20 pmol of each forward and reverse primer and 0.5 U of *Taq* DNA polymerase. PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the following conditions: initial denaturation at 94°C for 3min, 40 cycles consisting of 94°C for 1min (denaturation), 60°C for 1min (annealing), 72°C for 1min (primer extension) and final extension 72°C for 10 min.

Fengycin D

The forward primer FEND1F (5'TTTGGCAG CAGGAGAAGTTT3') and reverse primer FEND1 R (5'GCTGTCCGTTCTGCTTTTTC3') were used for amplification of fengycin gene (964 bp) (Athukorala *et al.,* 2009). The 20 µl mixture contained approximately 50 ηg of total DNA, 5 mM each dNTPs, 20 pmol of each forward primer and reverse primer and 0.5 U of *Taq* DNA polymerase. PCR amplification was performed in a thermocycler (Eppendorf Master cycler, Germany) using the conditions: initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension 72°C for 10 min.

Surfactin

The forward primer SUR3F (5'ACAGTATGGAGGCATGGTC3') and reverse primer SUR3R (5'TTCCGCCACTTTTTCAGTTT3') were used for amplification of surfactin gene (441 bp) (Ramarathnam *et al.,* 2007). The 40µl PCR reaction mixture contained DNA template 50ηg, 1X Taq buffer, 0.2 mM of each of dNTP mixture, 1µM of each primers, 1.5 mM $MgCl_2$ and 2U of *Taq* DNA polymerase. PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) of using the conditions: Initial denaturation at 94°C for 3min, 40 cycles consisting of 94°C for 1min (denaturation), 57°C for 1min (annealing), 72°C for 1min (primer extension) and final extension 72°C for 10min.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to check the quality of DNA and also to separate the products amplified through polymerase chain reaction. The sizes of the PCR products were determined by comparison with standard 100bp molecular marker (Bangalore Genei Pvt. Ltd., Bangalore, India). The PCR products were resolved on 1.5 per cent agarose with ethidium bromide (0.5µgml^{-1}), photographed and analyzed using gel documentation system (Alpha Innotech Corporation, San Leandro, California).

Testing crude antibiotics of B. amyloliquefaciens against Colletotrichum lindemuthianum

The crude antibiotics of *Bacillus* spp was extracted as per the protocol described by Prapagdee *et al.* (2012) with slight modifications. Bacterial cells were cultivated in Nutrient broth (NB) and incubated at 28°C for 3d. The supernatant was collected at 72 h (stationary phase) by centrifugation at 8,000 rpm for 30 min. Then supernatant was adjusted to acidic pH

2.0 by adding with concentrated HCl and the mixture was stirred at 100 rpm in an orbital shaker for 8hr. Antifungal compounds in supernatant or culture broth were extracted by adding the equal volume of solvent ethyl acetate and shaken vigorously for 1-2 h. Culture broth was extracted twice with ethyl acetate solvent for complete extraction. The solvent fraction that contained antifungal compounds were combined and concentrated by evaporation in the rotary flash evaporator maintained at 60°C, at 80rpm. The concentrated crude extract of the extracellular antifungal compounds was then dissolved in 1 ml methanol: chloroform mixture (1:1) for *in vitro* antifungal activity assay and GC-MS analysis.

The agar well diffusion assay, as reported by by Islam *et al.,* (2012) was used to determine the antagonistic activity of crude antibiotics from B. amyloliquefaciens. The PDA medium (20 mL) was poured into each sterile Petri plate, followed by placement of mycelial disc (5 mm in diameter) of the C. lindemuthianum at the center of the plates. A well (7 mm in diameter) was made by punching the agar with a sterile steel borer on the corner of the plate in four places with equal distance. Then crude antibiotics from B. amyloliquefaciens were poured into the wells separately at the rate of 100µl per well and incubated for 72 h at 28±2˚C. Three replications were maintained. The inhibitory activity was expressed as the percent growth inhibition, compared to the control (solvent only used in the wells), according to the following formula:

Growth inhibition (%) = (DC − DT)/DC × 100. where, DC, diameter of fungal colony in control; and DT, diameter of fungal colony with treatment (Pandey et al., 1982).

GC-MS analysis

Detection of active bio-molecules present in the crude antibiotics of *B. amyloliquefaciens* responsible for the suppression of *C. lindemuthianum* were carried out through GC-MS (GC Clarus 500 Perkin Elmer). Volatile compounds were identified by GC/MS using a coloumn Elite-5MS (100% Dimethyl poly siloxane), 30 x 0.25 mm x 0.25µm df equipped with GC clarus 500 Perkin Elmer. The turbo mass-gold-perkin-Elmer detector was used. The carrier gas flow rate was 1 ml per min, split 10:1, and injected volumes were 3µl. The column temperature was maintained initially at 110°C at the rate of 10°C/min-No hold followed by increases up to 280°C at the rate of 5° C /min-9 min (hold). The injector temperature was 250°C and this temperature was held constant for 36 min.

The electron impact energy was 70 eV, Julet line temperature was set at 2000°C and the source temperature was set at 200°C. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 aMU range. Using computer searches on the NIST Ver.2005 MS data library and comparing the spectrum obtained through GC/MS compounds present in the crude sample were identified.

Development of liquid formulation

B. amyloliquefaciens was cultured on Luria Bertani medium poured on to the sterile Petri Plate @ 15ml/plate. The medium was allowed to solidify and it was streaked with the stock culture maintained in NA broth amended with 40 per cent glycerol at - 80°C, using a sterile cotton buds. The plates were incubated at 28±2°C for 48hr. From these plates, a loop full of B. amyloliquefaciens was inoculated into 1000 ml of nutrient broth using the inoculation needle made up of nichrome wire and incubated in an orbital shaker at 150 rpm at room temperature (28±2°C) for 48hr. Later the liquid biomass along with bacterial cells for isolate was mixed with one per cent glycerol (10ml), tween 20 (10ml) and poly vinyl pyrrolidone – 40000 ml.wt (10g). The resultant mixture was kept at orbital shaker at 200 rpm for five minutes to ensure uniform blending and homogenization of the bacterial cells. Subsequently, cfu were assessed in nutrient agar medium using the serial dilution technique. One ml of the formulation consists of 2.5 ×1010 cfu/ml. (Dheepa et al., 2016).

Shelf life

The population present in the liquid formulation of *B. amyloliquefaciens* was assessed by serial dilution technique using 10^{-7} dilution. Three replications were maintained. The population of bacteria in the product was assessed upto 120 d at 15 d interval. The initial population was expressed upto 92.84 × 10^{8} cfu/ml which was gradually decreased to 3.24 × 10^{8} cfu/ml.

Bio-efficacy of liquid formulation of B. amyloliquefaciens against mungbean anthracnose

Glasshouse experiment was conducted to evaluate the efficacy liquid formulation of *B.amyloliquefaciens* against mungbean anthracnose along with chemical check (carbendazim). The experiment consisted of four treatments and five replications and treatments arranged in Completely Randomized Block Design (CRD), Four pots were maintained for each replication. The pathogen was artificially inoculated with the susceptible cultivar. The cultivar used was SML 1082. The disease incidence was assessed as per the standard grade chart furnished by Mayee and Datar, (1986). The treatment details are mentioned below.

Treatment details	
T_{1} -	Seed treatment with liquid formulation of B. amyloliquefacians @ 10ml kg^{-1} of seed
T_{2} -	T_{1} + foliar application of B. amyloliquefacians @ 10ml/litre of water at 25 DAS
T_{3} -	Seed treatment @ 2g kg^{-1} + foliar spray with carbendazim 0.1% at 25 DAS
T_{4} -	Untreated Control

Results and Discussion

A total of eight *Bacillus viz*., *B. amyloliquefaciens, B. altitudinus, B. licheniformis, B. endophyticus, B. sonorensis, B. aryabattai, B subtilis* CaB_5 and *B . subtilis* CcB_7 obtained from the culture collection at the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore were used for

Table 1. *In vitro* efficacy of *Bacillus* spp against *C. lindemuthianum*

Biocontrol agent	Mycelial growth (cm)*	Per cent reduction over control
B. licheniformis	4.67[b]	48.14[b] (43.93)
B. altitudinus	5.93[f]	34.8[f] (35.71)
B. amyloliquefaciens	4.07[a]	54.80[a] (47.76)
B. endophyticus	5.30[c]	41.11[c] (39.87)
B. sonorensis	5.57[d]	38.14[d] (38.13)
B. aryabattai	5.80[e]	35.55[e] (36.59)
B subtilis CaB_5	6.00[g]	33.33[g] (35.26)
B subitilis CcB_7	5.63[de]	37.40[de] (37.70)
Control	9.00[h]	0.00

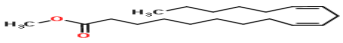
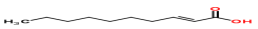
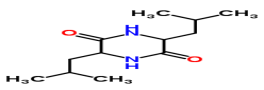
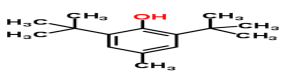
*Values are means of three replications; Means in a column followed by same superscript are not significantly different according to DMRT at 5% level. Values in parenthesis are arcsine transformed values

evaluating their antagonistic acti vity against *C. lindemuthianum* (Table 1). All the Bacillus strains exhibited more than 30 per cent growth inhibition. Among these strains, *B. amyloliquefaciens* recorded the average mycelial growth of 4.07cm with 54.8 per cent inhibition. It was followed by *B. licheniformis* which showed mycelia growth of 4.67 with 48.14 per cent growth inhibition. The highest mycelial growth of 6.0 cm and lowest growth inhibition of 33.33 was exhibited by *B. subtilis* CaB_5. *B. japonicum* and *B. subtilis* exhibited very broad spectra of action with an efficient antagonistic activity against *F. culmurum* and *B. cinerea* by 40.79 and 37.61% of mycelial growth inhibition. The inhibition of fungal growth is due to secretion of diffusible and volatile compounds by *B. subtilis* (Chaurasia et al., 2004). *Bacillus* spp are recognized as safe biocontrol agents specifically as seed protectants and antifungal agents. The *Bacillus* species has been in the spotlight for biocontrol research for decades by various modes of actions (Jamalizadeh *et al.,* 2011).

Growth promotion activity of Bacillus isolates

All the eight *Bacillus* spp treated with the mungbean seeds showed enhanced growth parameters over untreated control. Among these, the *B. amyloliquefaciens* showed the higher vigour index of 3026.78 and it was on par with *B. endophyticus* with vigour index 2971.41. It was followed by

Table 2. Antibiotics identified in ethyl acetate extract of *B. amyloliquefacians* by GC-MS analysis

Compounds	Retention time	Area (%)	Molecular Formula	Molecular structure	Mode of action
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	18.79	31.12	$C_{19}H_{34}O_2$		Antifungal
6-Octadecenoic acid, methyl ester, (Z)-	18.99	14.16	$C_{19}H_{36}O_2$		Antifungal
Hexadecanoic acid methyl ester	14.11	7.72	$C_{17}H_{34}O_2$		Antifungal
Trans-2-Decenoic acid	7.48	7.43	$C_{10}H_{18}O_2$		Antifungal
Methyl stearate	19.86	1.78	$C_{19}H_{38}O_2$		Antifungal, Antioxidant
2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	21.06	1.67	$C_{12}H_{22}N_2O_2$		Antifungal
Butylated Hydroxytoluene	8.30	4.21	$C_{15}H_{24}O$		Antifungal
2-Dodecenoic acid	8.97	0.63	$C_{12}H_{22}O_2$		Antifungal
n-Hexadecanoic acid	14.91	1.55	$C_{16}H_{32}O_2$		Antifungal
2-Myristynoic acid	8.82	0.94	$C_{14}H_{24}O_2$		Antifungal

B. licheniformis which recorded the vigour index 2959.11. The lowest vigour index of 2389.01 was exhibited by *B. alitudinus* (Fig 1). Growth promotion and disease control by *Bacillus* are complex interrelated processes involving direct and indirect mechanisms that include the synthesis of some metabolites (auxin, cytokinins and gibberellins), induction of ACC deaminase, production of siderophore, antibiotics, HCN and volatile compounds, mineral solubilisation, competition and ISR (Lazarovits and Nowak, 1997).

Table 3. Effect of liquid formulation of *B. amyloliquefacians* on growth and yield of mungbean

Treatment. No	Treatment	Per cent disease Incidence (PDI)	Per cent reduction over control	Plant height (cm)*	No of pods/ plant*	No of seeds/ plant*	Yield. plant^{-1}* (kg/ha)
T_1	Seed treatment with liquid formulation of *B. amyloliquefacians* @ 10ml kg^{-1} of seed	37.49^c (37.78)	44.45	57.60^c	10.31^c	7.72^c	7.78^c
T_2	T_1 + foliar application of *B. amyloliquefacians* @ 10ml/ litre of water at 25 DAS	12.25^a (25.27)	62.05	67.34^a	10.27^a	9.20^a	4.73^a
T_3	Seed treatment @ 2g kg^{-1} + foliar spray with carbendazim 0.1% at 25 DAS	13.83^b (21.91)	79.39	62.20^b	11.43^b	8.10^b	3.06^b
T_4	Untreated Control	67.59^d (55.29)	0	45.70^d	6.00^d	6.45^d	1.80^d

*Values of mean of four replications; Means in a column followed by same superscript letters are not significantly different according to DMRT at 5% level. Values in parentheses are arcsine transformed values

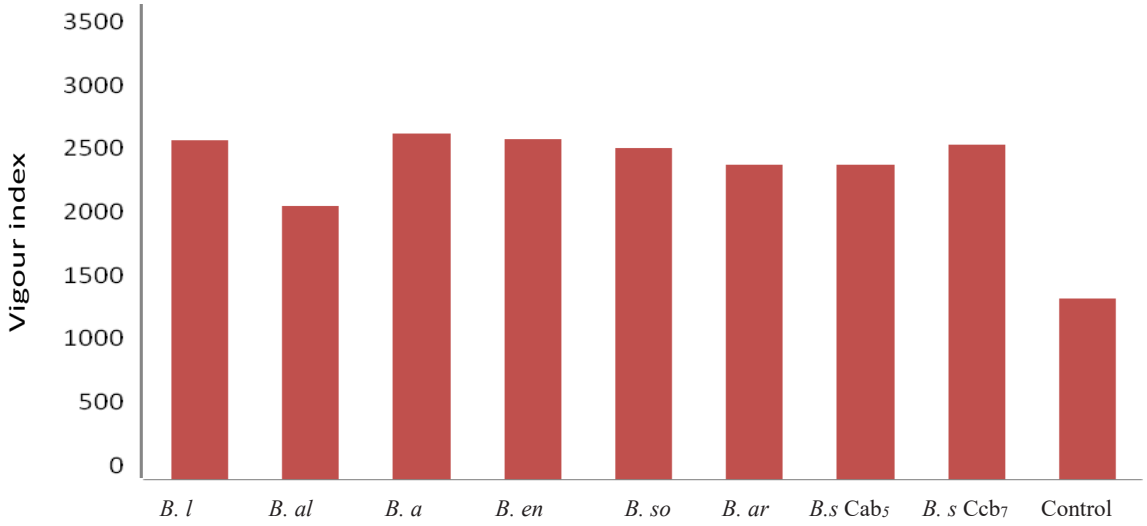
Detection of lipopeptide antibiotic genes of Bacillus strains

To find out the presence of antibiotic biosynthetic genes, the gene specific region of Iturin A, Fengycin D and Surfactin were amplified with the antibiotic specific primers for Iturin A (ITUD1F and ITUD1R), Fengycin D (FEND1F and FEND1R) and Surfactin (SUR3F and SUR3R).

Amplification of iturin A gene

Among the eight *Bacillus* spp tested for the presence of Iturin A gene, six *Bacillus* strains viz., *B. altitudinus, B. amyloliquefaciens, B. endophyticus, B. sonorensis, B. aryabattai* and *B. subtilis* CaB_5 showed an amplified gene product of 647 bp, which indicated the presence of Iturin A gene. Remaining

Fig 1. Growth Promoting activity of Bacillus spp on mungbean seedlings (Roll towel method)



B. l – B. licheniformis

B. al – B. altitudinus

B. am – B. amyloliquefacians

B. e – B. endophyticus

B.sol – B. sonorensis

B. ar – B. aryabattai

B. s Cab_5 *– B. subitilis* Cab_5

B. s Ccb_7 *– B. subitilis* Ccb_7

two strains *viz*., *B. licheniformis* and *B. subtilis* CcB_7 not showed amplification of Iturin A gene (Plate 1a). The ability of *B. amyloliquefaciens* to produce iturin has previously been reported by Arrebola *et al*., (2010). Iturin A produced by *B. amyloliquefaciens* PPCB004 affected the mycelial growth of several fungal pathogens (Arrebola *et al.,* 2010). The *B. subtilis* culture tested positive for the presence of iturin antibiotic gene (Smitha *et al.,* 2017).

Amplification of fengycin D gene

The specific primer for the Fengycin D bind with the genomic DNA and amplified to form amplicon of size 964 bp. All the eight strains of *Bacillus* spp tested showed the presence of Fengycin D gene (Plate 1b). Fengycins are less haemolytic than iturin and surfactin but have strong fungitoxic activity specifically against filamentous fungi (Rajesh *et al.,* 2014).

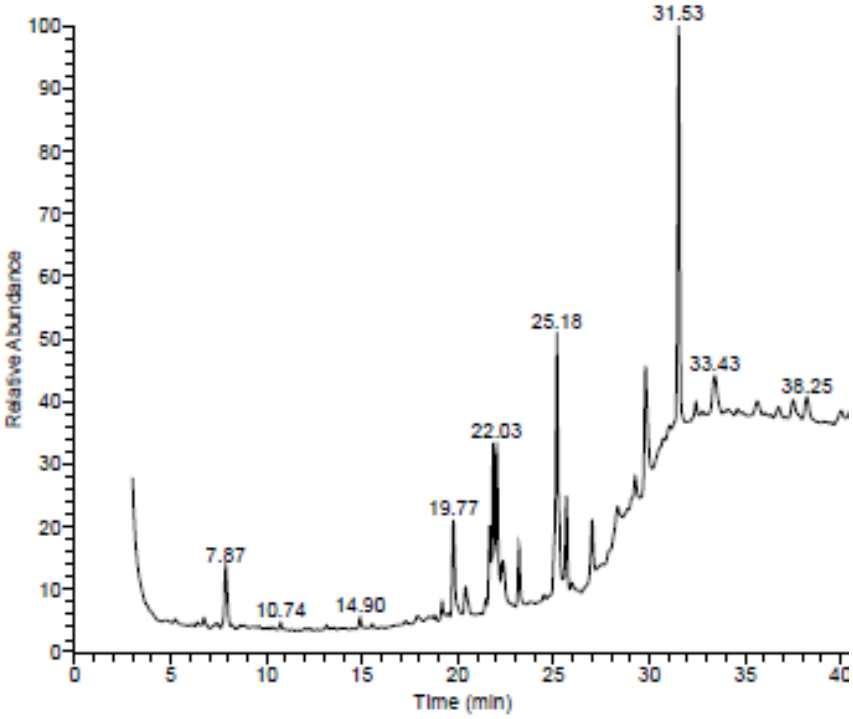


Fig 2. A Chromotogram of GC-MS analysis of crude metabolities of *B. amyloliquefaciens*

Amplification of surfactin gene

Out of eight strains, seven strains *viz*., *B. altitudinus, B. amyloliquefaciens, B. endophyticus, B. sonorensis, B. aryabattai* and *B. subtilis* CaB_5 amplified with the amplicon size of a 441 bp, which indicated the presence of surfactin gene (Plate 1c). The surfactin gene was not detected in *B. licheniformis*. Surfactins are the non-ribosomally synthesised cyclic lipopeptide metabolite of *Bacillus* spp. The surfactin family comprises of surfactin and lichenysins. Similar results earlier reported by many workers (Vinodkumar *et al.,* 2017; Smitha *et al.,* 2017; Ji, 2013).

GCMS analysis of secondary metabolities of B. amyloliquefaciens

The antimicrobial biomolecules present in the effective strain of *B. amyloliquefaciens* was analyzed through the GCMS using crude antibiotics at Agricultural College & Research Institute, Madurai. The GC-MS analyses results showed the presence of 10 different antimicrobial compounds in crude antibiotics. The major antifungal compound present in the extracts were 9,12-Octadecadienoic acid (Z,Z)-, methyl ester with an area of 31.12 per cent. Hexadecanoic acid methyl ester with an area of 7.72 per cent, Butylated Hydroxytoluene with an area of 4.21 per cent and n-Hexadecanoic acid with an area of 1.55 per cent (Fig 2, Table 2). This result was in agreement with findings of Cordovez *et al.* (2015). Gas chromatography is a widely applied technique in many branches for the identification of volatile compounds from the *B. amyloliquefaciens* culture broth and it is very reliable to identify the compound in complex biochemical product. Many workers characterized antibiotics of *Bacillus* through GC-MS analyses (Vinodkumar *et al.,* 2017; Ji, 2013; Arrebola *et al.,* 2010).

Bioassay against Colletotrichum lindemuthianum

The crude antibiotics extracted from the *B. amyloliquefaciens* were tested for their antifungal action against *C. lindemuthianum* by agar well diffusion assay. The results revealed that the crude antibiotics extracted from *B. amyloliquefaciens* were found to be promising in reducing the mycelial growth of *C. lindemuthianum* by 53.6 per cent. The success of biocontrol agent depends on its ability to produce

antimicrobial compounds. The lipopeptide antibiotics produced by *B. subtilis* were generally assumed to be responsible for the biocontrol activities. *B subtilis* strain AB_{14} showed antagonistic activity against *C. acutatum* (Lamsal *et al*., 2012). Sclerotial formation was completely inhibited by *B. amyloliquefaciens* strains VB_2, VB_6, and VB_7. *B. subtilis* strains BSC_7 and VB_{10} showed the inhibition of sclerotial production, the strains also reduced the density of mycelia compared to the healthy control (Vinod kumar *et al.,* 2017). Crude extract of *B. amyloliquefaciens* highly affect all fungal pathogens growth with 8.6, 6.0, 5.3 and 3.0 mm of zone inhibition against *P. capsici, B. cinerea, F. oxysporum* and *C. gloeosporioides* respectively. Additionally, various concentrations of crude extract of *B. amyloliquefaciens* Y1 culture showed significant antifungal activity against all tested fungal plant pathogens. This prove that crude extract of *B. amyloliquefaciens* Y1 have secondary metabolites which inhibit growth of fungi. (Jamal *et al.,* 2015)

Plate 1a. PCR amplification Iturin A gene of *Bacillus* spp

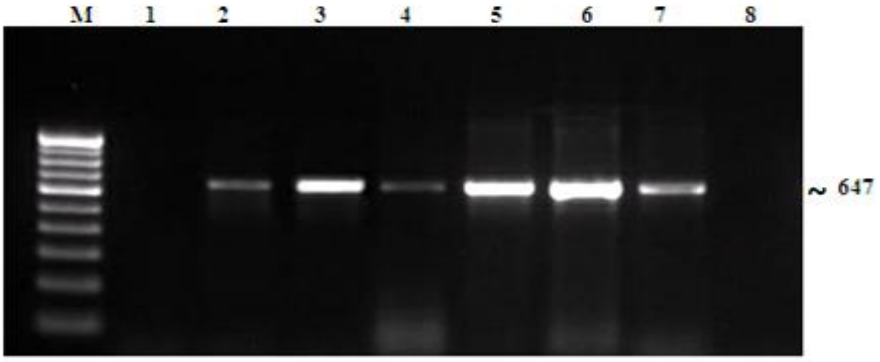


Plate 1b. PCR amplification Fengycin D gene of Bacillus spp

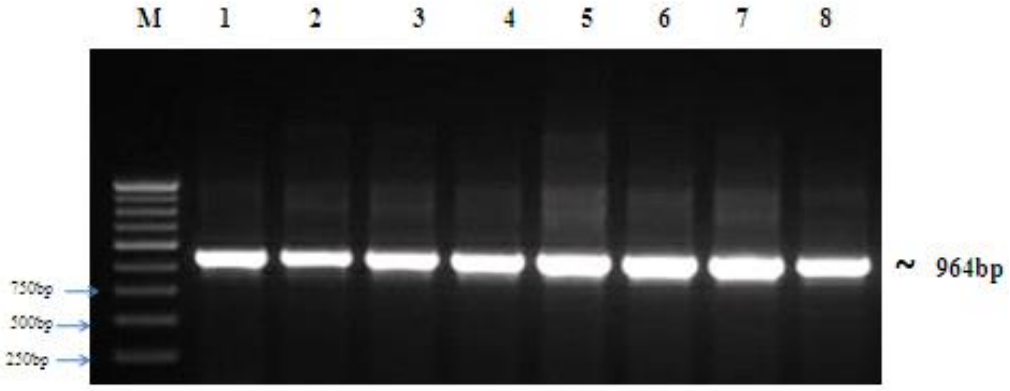
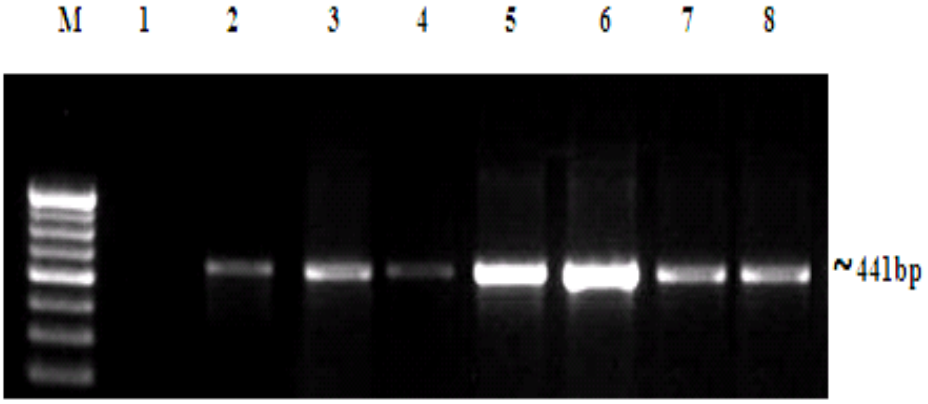


Plate 1c. PCR amplification Surfactin gene of Bacillus spp



M – 100 bp ladder
1 – *B. licheniformis*
2 – *B. altitudinus*
3 – *B. amyloliquefacians*
4 – *B. endophyticus*
5 – *B. sonorensis*
6 – *B. aryabattai*
7 – *B. subitilis Cab5*
8 – *B. subitilis Ccb7*

Shelf life of liquid formulation of B. amyloliquefaciens

A pot culture experiment was conducted to determine the efficacy of liquid formulation of *B. amyloliquefaciens* on the incidence of mungbean anthracnose. Seed treatment with liquid formulation of *B. amyloliquefaciens* @ 10ml/kg + foliar spray @ 10ml/litre of water at 25 DAS was the best treatment by recording the PDI of 12.25 per cent with 62.05 per cent disease reduction. The chemical check *viz.,* carbendazim (seed treatment @ 1g kg^{-1} of seed + foliar spray @ 0.1%) reduced the disease incidence by 79.39 per cent. The treatment T_2, showed highest plant height of 67.34 cm which are significantly superior over control (T_4) which recorded the plant height of only 45.70 cm. The same treatment T_2 also registered the plant yield of 4.73g, pod number (10.27 pods/plant), seeds number (9.2 seeds/pod). Seed treatment with carbendazim + folair spray (T_3) recorded the plant height 62.2 cm with per plant yield of 3.06 g. The untreated control recorded the lowest number of pods/plant (6), seeds/pod (6.45) and the yield/plant (1.80g) (Table 3). *Bacillus* species have become attractive biological control agents due to their ability to produce hard, resistant endospores and antibiotics which control a broad range of plant pathogens (Cao *et al.,* 2018). The anthracnose incidence in chilli caused by *C. capsici* was reduced when pathogen inoculated plants were treated with *B. subtilis.* Bacterized seeds significantly reduced the percentage of anthracnose plants from 99 to 60 per cent and also reduced foliage yellowing and disease severity (Zaim *et al*., 2013).

The most promising isolate *B. amyloliquefaciens* was selected based on their *in vitro* antagonism against *C. lindemuthianum* and plant growth promotion. The selected strain was formulated in the liquid formulation and the initial population in the formulation was assessed to be 92.84 x 10^8cfu/ml. The population of *B. amyloliquefaciens* in the formulation was assessed at 15 d intervals for a period of 120 d. The population of *B. amyloliquefaciens* was 3.24 x 10^8 cfu/ml at 120 d after incubation. This was in line with the findings of Dheepa *et al.* (2016). Endospores are also more resistant to drying processes for liquid formulation and are relatively easy to produce with industrial fermentation technology (Brannen and Kenney, 1997).

Conclusion

Multifaceted bacterial antagonist *B. amylolique faciens* is capable of producing a wide variety of secondary metabolites. Crude extracts of *B. amyloliquefaciens* reduced mycelial growth of *C. lindemuthianum.* The production of antibiotic compounds highlights *B. amyloliquefaciens* as a good candidate for the development of biocontrol agents.

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