Acid Tolerant Bacterium *Bacillus Amyloliquefaciens* MBNC Retains Biocontrol Efficiency Against Fungal Phytopathogens in Low pH

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Abstract

Soil pH conditions have important consequences for microbial community structure, their dynamics, ecosystem processes, and interactions with plants. Low soil pH affects the growth and functional activity of bacterial biocontrol agents which may experience a paradigm shift in their ability to act antagonistically against fungal phytopathogens. In this study, the antifungal activity of an acid tolerant soil bacterium *Bacillus amyloliquefaciens* MBNC was evaluated under low pH and compared to its activity in neutral pH conditions. Bacterial supernatant from three-day old culture grown in low pH conditions were more effective against fungal pathogens. *B. amyloliquefaciens* MBNC harboured genes involved in the synthesis of secondary metabolites of which surfactin homologues, with varying chain length (C11 – C15) were identified through High-Resolution Mass Spectroscopy. The pH of the medium influenced the production of these metabolites. Surfactin C15 was exclusive to the extract of pH 4.5; production of iturinA and surfactin C11 was detected only in pH 7.0 while, surfactin C12, C13 and C14 were detected in extracts of both the pH conditions. The secretion of phytohormones viz. Indole Acetic Acid (IAA) and Gibberellic Acid (GA) by *B. amyloliquefaciens* MBNC were detected in higher amount in neutral condition compared to acidic condition. Although, secretion of metabolites and phytohormones in *B. amyloliquefaciens* MBNC was influenced by the pH condition of the medium, the isolate retained its antagonistic efficiency against several fungal phyto-pathogens under acidic condition.

Introduction

Plant diseases caused by pathogens is an important factor limiting increase of crop yield. The intensive agricultural production system practiced globally relies on the use of agrochemicals for crop protection. Unfortunately, over-dependence and their indiscriminate use have led to the development chemical resistant pathogen and impacted our environment including affecting on non-target organisms and loss of biodiversity (de Weger et al. 1995). The pesticide residues left in the soil also make their way into the food chain system and affect human health (Zhang et al. 2011). The use of microbial biological control agents (MBCAs) to control the infestation of crop pest and disease offer a sustainable alternative for crop production. The MBCAs exert their antagonistic effect by secreting inhibitory metabolites, compete with the pathogenic organism for nutrients or space, induction of systemic resistance in plants (Aeron et al. 2011; Saraf et al. 2014). However, the MBCAs released in the crop field encounter several abiotic stress conditions including low pH, salinity, temperature fluctuations, osmotic and oxidative stresses, availability of nutrients and water. These factors not only influence the growth and survivability of the applied MBCAs but also their antagonistic efficiency. The success of the MBCAs in suppressing the target pathogen is determined by its ability to tide over the stress condition while retaining its viability and efficacy. An understanding of the efficacy of the potential MBCAs in terms of its environmental fitness is thus, important before they are released for commercial purpose. Very few studies have been directed towards understanding the effects of low pH condition on the effectiveness of biocontrol agents.

Soil acidity has impact on several agricultural process including influencing soil proprieties and processes that affect nutrients solubility and their availability for plant growth (Gentili et al. 2018). Soil pH is regarded as the most important driver for soil microbial community structure and their function (Goswami et al. 2017; Ratzke and Gore 2018) as pH of the soil nearing neutral or varying towards alkaline conditions promote bacterial

growth while acid pH favour fungal growth. The MBCAs are therefore likely to experience a paradigm shift in their ability to act antagonistically against certain phytopathogens depending on their ability to survive and suppress the growth of the pathogen. Fungi adapt well in acidic condition and are reported to grow fivefold faster in acidic condition while the bacterial growth is inhibited under the same condition and decreases by a similar fold (Rousk et al. 2009). Under acidic condition, the probability of the fungal pathogen becoming aggressive is high due to stifled activity of antagonistic bacteria. Low acidic soil conditions (pH 4.5–5.5) is reported to suppress the growth and antagonistic activity of *Bacillus cereus* and *Pseudomonas fluorescens* while favouring the growth of the wilt causing pathogen, *Ralstonia solanacearum* (Li et al. 2017).

Previous studies have shown that the efficiency of growth promoting activity of plant growth promoting bacteria (PGPBs) decreases in acid stress conditions (Goswami et al. 2018). In an attempt to assess whether acidic conditions also hinder the antagonistic efficiency of MBCAs, we took a previously isolated acid tolerant isolate of *Bacillus amyloliquefaciens* MBNC (Chowdhury et al. 2021) to evaluate its ability to act against six plant pathogenic fungi under acid stress condition. The spore-forming bacteria, *B. amyloliquefaciens* is widely distributed in nature and is well reported for its biological control activity against several plant pathogens (Romero et al. 2007). The bacteria exert its antagonistic effect mainly through the production of a broad range of antimicrobial compounds like lipopeptides, antibiotics and siderophores (Park et al. 2001; Benitez et al. 2010; Shafi et al. 2017). The lipopeptides such as surfactin, iturin, and fengycin produced by the bacteria are reported to act antagonistically towards several pathogens (Chen et al. 2009). Additionally, *B. amyloliquefaciens* is also reported to promote plant growth through secretion of phytohormones, solubilising minerals and promote soil aggregation (Qaiser et al. 2018; Deka et al. 2019).

Materials And Method

Fungal pathogens and their taxonomic characterization

Six phytopathogenic fungal isolates were collected from the Department of Plant Pathology, Assam Agricultural University, Assam which were identified as *Pythium myriotylum* 1NC, *Fusarium verticilloides* 2NC (synonym. *Fusarium moniliforme*), *F. incarnatum* 3NC, *Athelia rolfsii* 4NC (synonym. *Sclerotium rolfsii*), *Fusarium oxysporum* 5NC, and *F. redolens* 6NC. However, for further confirmation, the isolates were subjected to molecular characterization through sequencing of the ribosomal Internal Transcribed Spacer (ITS) region. The ITS region of the fungal genome was amplified with a pair of universal primer: ITS1 and ITS4 (White et al. 1990) (Table 1). The PCR thermal profile was as follows: initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s (35 cycles), annealing at 50 °C for 30 s, extension at 72 °C for 45 s, and a final extension step at 72 °C for 7 min. The PCR amplicons were purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, USA) and the purified products were sequenced (Bio-Serve Biotechnologies, India). The sequence reads obtained post sequencing were assembled. A similarity search was employed using BLAST and compared with references at GenBank, NCBI to obtain their identity. Further, a phylogenetic tree was constructed from the ITS sequence data using MEGA 6.0 (Tamura et al. 2013). Sequences were aligned using clustalW and the Maximum Likelihood tree was prepared with Tamura-Nei model employing 1000 bootstrap replications.

Primary Screening for antifungal activity of *B. amyloliquefaciens* MBNC

Dual culture disc diffusion method was used for confirming antifungal activity of the acid tolerant isolate *B. amyloliquefaciens* MBNC. The culture plates were divided into two halves with a line through the diameter of each plate. Mycelial agar blocks (5-mm-diameter) of fungal phytopathogens were placed onto one half of the culture plate (15mm away from the centre) and allowed to grow in individual plates for two days. The isolate *B. amyloliquefaciens* MBNC was inoculated in 50 ml Nutrient broth (NB) and incubated overnight at 37 °C, in shaking (180 rpm). This overnight grown culture was used for soaking sterile disc of filter paper (15 mm diameter), which were further placed at the opposite half of each culture plates containing the individual fungal pathogens. The plates were incubated at 28 °C until inhibition of fungal mycelia was observed.

Effect of pH on antagonistic activity of *B. amyloliquefaciens* MBNC

The effect of pH on the antifungal activity of *B. amyloliquefaciens* MBNC was assessed using dual culture method at pH 4.5 and pH 7.0. Spot inoculation of *B. amyloliquefaciens* MBNC was done onto one half of the culture plate, while the other half was inoculated with each of the fungal pathogens. For obtaining the desired neutral and acidic pH, the pH of the PDA medium was adjusted with sodium hydroxide and tartaric acid, respectively.

Preparation of bacterial supernatant

Bacillus amyloliquefaciens MBNC was inoculated in 50 ml Nutrient broth (NB) and incubated overnight at 37 °C, in shaking (180 rpm). One millilitre of the overnight culture was then used to inoculate fresh NB adjusted to pH 7.0 and pH 4.5 and incubated at 37°C with shaking (180 rpm). For each pH, seven different NB was used as representative for seven days (Day 1 to Day 7). Twenty millilitre culture of each pH was then transferred to a 50-ml tube, and the culture filtrate was obtained by centrifugation at 8000rpm for 15 min at 4°C. The supernatant was filtered through a 0.22-µm membrane filter into sterile tubes and kept at 4 °C until further use. This procedure was repeated consecutively for each day starting from Day 1 to Day 7.

Evaluation of antifungal activity

An aliquot of 100µl sterile bacterial supernatant (previously stored in 4 °C), each of pH 7.0 and pH 4.5 was embedded/spread on PDA plates and allowed to dry. This was followed by placing of a 5-mm-diameter mycelium agar block of each of the fungal isolates at the centre of individual plate and incubated in 28 °C incubator. The difference in fungal mycelia growth diameter at both pH condition was recorded consecutively for 7 days and calculated as inhibition percentage. The relative inhibition rate was calculated as follows:

Relative inhibition (%) = $[(R_1 - R_2)/R_1] \times 100$ where, R_1 is the colony diameter of control (fungal colony grown on PDA plates without the presence of any bacterial supernatant) and R_2 is the diameter of fungal colony grown on PDA plate embedded with filter sterilized bacterial supernatant grown in either pH 7.0 or pH 4.5.

This procedure was repeated for the supernatant obtained from the cultures of Day 1 to Day 7. But since the bacterial supernatant obtained from the cultures grown for three days showed higher inhibition against all the fungal phytopathogen (data not shown), only the Day 3 supernatant has been taken into consideration for further studies.

Heat stability test

To evaluate the heat labile nature of the secondary metabolites, cell free supernatant of *B. amyloliquefaciens* MBNC was exposed to 100 °C in a water bath for 1 h (Mariam et al. 2014). The cell free supernatant was loaded into wells of PDA plates having 24h-old culture of the fungal pathogens. Inhibition of mycelial growth near the punched well containing the heat-treated culture supernatant confirmed the heat stability of the antifungal compounds in the culture supernatant.

Detection of secondary metabolite biosynthetic genes

Eight genes viz. *srfA* (surfactin biosynthetic gene) *dfnD* (gene for difficidin biosynthesis), *fenA* (gene for fengycin biosynthesis), *baeR* (gene for bacillaene biosynthesis), *ituA* (gene for iturin A), *bacD* (gene for bacilysin biosynthesis), *dhbE* (gene for bacilibactin biosynthesis) and *mlnA* (gene involved in biosynthesis of macrolactins) belonging to the different secondary metabolite biosynthetic pathways (Dunlap et al. 2013) were selected for screening through PCR based method. Genomic DNA from isolate *B. amyloliquefaciens* MBNC was used as template for PCR amplification of the secondary metabolite biosynthetic genes. The PCR reaction was performed using EmeraldAmp® GT PCR Master Mix (Takara, Japan) in 50 µl reaction volume containing 10 pmol of each primer (Table 1) and 50ng of genomic DNA. The PCR condition was initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 45 s (35 cycles), annealing temperature for 30 s, extension at 72 °C for 1.5 min, and a final extension step at 72 °C for 7 min. The amplified products were analysed on a 1.2% agarose gel.

Extraction of metabolites from culture supernatant of *B. amyloliquefaciens* MBNC

Preparation of bacterial supernatant was carried out using the procedure mentioned earlier (see Preparation of bacterial supernatant section) followed by filtration through Whatman filter paper No. 2. Extraction of culture supernatant was carried out according to method described by Burianek and Yousef (2000) with slight modifications. The filtrate of the culture supernatant was acidified with 1 N HCl to pH 3.0. An equal volume of chloroform was mixed with the culture supernatant (1:1, v/v) followed by vigorous stirring for 20 min. The organic fraction that was soluble in chloroform was separated into a clean round bottom flask and concentrated under vacuum. This organic extract was used as crude extract and was dissolved in specific solvent according to requirements for further analysis. For confirming the antifungal activity of the extracted supernatant, the crude extract (extract of both pH 7.0 and pH 4.5) was dissolved in dimethyl sulphoxide (DMSO) and tested against the fungal pathogens by well diffusion method.

Identification and characterization of bioactive antifungal components produced under normal and acidic condition

To determine the extracellular active metabolites in the bacterial supernatant the concentrated chloroform extract was re-dissolved in acetonitrile at a concentration of 100 µg/ml. The extract was then filtered through 0.22 µm PVDF membrane syringe filter (GE healthcare, USA) and 10 µl of filtrate was subjected to HRMS analysis in a Xevo G2-XS QT of HRMS system (Waters, USA). The positively ionized adduct ions of different compounds were calculated manually and compounds were identified by comparing them with the molecular information in PubChem database of NCBI (Maryland, USA).

Transcriptional regulation of *srfA* and *ituA* under normal and acid stress conditions

Total RNA was extracted from *B. amyloliquefaciens* MBNC from cultures at pH 7.0 and pH 4.5, grown for one (Day 1) and three days (Day 3), using Purelink™ RNA mini kit (Ambion, Life Technologies, USA) following the manufacturer's instructions. The first strand cDNA synthesis was carried out using 2 µg total RNA in 50 µl reaction using Proto Script cDNA synthesis kit (Biolab, Life Technology) in a thermal cycler (ABI, USA). Based on the detection of bioactive metabolite in HR-MS analysis, expression profiles of the genes *srfA* and *ituA* were quantified by quantitative real-time PCR (qRT-PCR) in a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA) in a total reaction volume of 20 µL containing 10nM of each primer and 50 ng cDNA templates. Primers used for real-time PCR study are listed in Table 1. The thermal program used was as follows: initial denaturation at 95 °C for 10 min followed by 35 cycles of 15 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C. The 16S rRNA gene was used as a reference gene (Goswami et al. 2018, Hazarika et al. 2019). The qRT-PCR runs were performed with three biological and technical replicates. The relative expression were evaluated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Evaluation of biocontrol potential of *B. amyloliquefaciens* MBNC

The effect of biocontrol potential of *B. amyloliquefaciens* MBNC was evaluated against six fungal phytopathogens during germination of bean (*Phaseolus vulgaris*) seeds. The bean seeds were surface sterilized with 1% sodium hypochlorite solution followed by 3 times washing with sterilized water. After that a suspension of bacterial cells was prepared in 0.85% saline solution and the cell numbers were adjusted to OD₆₀₀ of 0.6 followed by soaking the bean seeds overnight in the bacterial suspension. For the control set, seeds were soaked overnight in 0.85% saline solution without any bacterial cells. The six fungal pathogens were cultured separately to prepare spore suspensions with approximately $\sim 10^4$ spores per ml as the initial concentration. The seeds (with and without bacterial treatment) were then exposed to each fungal spore suspensions separately for 30 min. A respective of eight seeds for each experimental condition were aseptically transferred to sterile petri plates lined with moistened filter paper. The experiment was carried out in triplicates. The germination percentage and root length were measured after 1 week of fungal treatment. Another plate containing surface sterilized seeds was kept as control to evaluate the normal germination efficiency of the seeds against the bacteria treated and untreated plates.

Screening plant growth promoting properties (PGP) of *B. amyloliquefaciens* MBNC

The bacterial isolates were screened for phytohormone production viz. Indole acetic acid (IAA) and gibberellic acid (GA) *in-vitro*. Quantitative estimation of IAA was performed by the method as described by Patten and Glick (2002). The isolate *B. amyloliquefaciens* MBNC was cultured overnight in DF salt minimal media (containing 4g KH₂PO₄, 6g Na₂HPO₄, 0.2g MgSO₄, 1µg FeSO₄, 10µg H₃BO₃, 10µg MnSO₄, 70µg ZnSO₄, 50µg CuSO₄ and 10µg MoO₃ with 0.2% glucose, 0.2% gluconic acid and 0.2% citric acid /1 litre) adjusted to pH 7.0 and pH 4.5. Twenty micro litres of the overnight grown bacterial culture were transferred respectively into 5 ml of DF salt minimal media (adjusted to pH 7.0 and pH 4.5), amended with filter sterilized L-tryptophan (1000 µg/ml) and incubated at 30°C for 72 h. this was followed by collecting the culture supernatant by centrifugation at 5,000 rpm for 10 min. For measuring the amount of IAA produced, 1ml of culture supernatant was mixed with 4 ml of Salkowski reagent (150 ml concentrated H₂SO₄, 250 ml of distilled H₂O, 7.5 ml 0.5 M FeCl₃.6H₂O solution). Development of pink colour indicates the production of IAA. After 20 min optical density was taken at 535 nm by UV-VIS spectrophotometer (Spectroquant 300, Merck, Germany). Concentration of IAA

produced by the bacterium was measured with the help of standard curve of IAA obtained in the range of 10-100 µg/ml. Quantitative estimation of GA was determined by the method described previously by Vikram et al. (2007). One ml of 0.6 OD fresh culture of *B. amyloliquefaciens* MBNC grown in pH 7.0 and pH 4.5 was inoculated in Nutrient broth amended with L-tryptophan (1mg/ml) and incubated at 30°C for 72 h. Five ml of the supernatant was taken in a test tube to which 0.4 ml of zinc acetate was added. After 2 minutes, 0.4 ml of potassium ferrocyanide was added and centrifuged at 1000 rpm for 15 min. To 3 ml of this supernatant, 3 ml of 30% HCl was added and incubated at 20°C for 75 min. The blank sample was treated with 5% HCl and the absorbance of the sample as well as blank was measured at 254 nm in a spectrophotometer (Spectroquant 300, Merck, Germany). The procedure was carried out with *B. amyloliquefaciens* MBNC cultured in both pH 7.0 and pH 4.5. The amount of GA present in the extract was calculated from the standard curve of GA and expressed as µg/ml of the medium.

Results

Identification of the fungal pathogens

Molecular characterization of the fungal isolates was carried out by sequencing the ITS regions followed by BLAST analysis to reveal all the six isolates as fungal phytopathogens. Sequencing of the ribosomal ITS region is regarded as one of the effective molecular tools for fungal identification (Schoch et al. 2012). Based on the BLAST similarity scores, the isolates were identified as *Pythium myriotylum* 1NC, *Fusarium verticilloides* 2NC (synonym. *Fusarium moniliforme*), *Fusarium incarnatum* 3NC, *Athelia rolfsii* 4NC (synonym. *Sclerotium rolfsii*), *Fusarium oxysporum* 5NC, and *F. redolens* 6NC. The phylogenetic tree constructed using the ITS sequences of the fungal pathogens revealed their close evolutionary relationships with the type species (Fig.1). The ITS sequences were submitted to GenBank and the accession number obtained are given in Table 2.

Antagonistic activity of *B. amyloliquefaciens* MBNC against fungal phytopathogens

A primary screening by disc diffusion method was used to detect antifungal activity of the isolate *B. amyloliquefaciens* MBNC against the six different fungal plant pathogens. It was observed that isolate *B. amyloliquefaciens* MBNC could inhibit the mycelial growth of all the six fungal pathogens under the test condition (Supplementary Fig. S1). A comparative study was further carried out using dual culture technique at both pH 7.0 and pH 4.5 to evaluate the effect of low pH on the antagonistic activity of *B. amyloliquefaciens* MBNC. The bacterium was able to inhibit the fungal pathogens at both acidic and neutral pH condition (Fig. 2). Highest inhibition of growth was recorded in case of *Fusarium verticilloides* at both pH 7.0 and pH 4.5. The inhibitory activity was higher at pH 4.5 against *F. oxysporum* and *A. rolfsii*, as compared to that of pH 7.0 (Fig. 2). The effectiveness of bacterial culture supernatant collected from liquid culture with pH 4.5 and 7.0 in suppressing fungal growth was also assessed. The bacterial supernatant of three-day old culture was found to be more effective in suppressing the growth of the fungal pathogens in comparison to the seven-day culture supernatant as evident from the maximum inhibition percentage (Table 3). The three-day old bacterial supernatant from acidic pH was more effective against the fungal pathogens compared to that of neutral pH with an exception in *F. verticilloides* showing 25.13±0.52% and 9.77±0.38% inhibition in pH 7.0 and pH 4.5, respectively. In contrast, the seven-day old bacterial supernatant from acidic pH showed less efficiency

against the fungal pathogens compared to that of neutral pH. Under the same condition, the inhibition was found to be negligible in case of *A. rolfsii* in both the pH (pH 7.0 and pH 4.5). The bacterial culture supernatant was ineffective against *A. rolfsii* due to the fast growth-rate of the fungus. In both the pH, the inhibition was found to be negligible in case of *A. rolfsii*. Initially, the inhibition zone was detected on the very next day of inoculation, which was subsequently replaced by the growing mycelia.

Heat Stability test of culture supernatant

The culture supernatant exposed to 100 °C did not lose its antagonistic activity as observed from the inhibition zone produced against each fungal phytopathogen (Supplementary table S1). Highest antagonistic activity was exhibited against *P. myriotylum* and *F. redolens* while lowest activity was observed against *F. verticilloides*. This indicated that the bacterium produced certain antifungal compound(s) which remain active at high temperature.

Antifungal activity of metabolites produced by *B. amyloliquefaciens* MBNC

The DMSO solubilized chloroform extracts collected from both pH 7.0 and pH 4.5 showed significant antifungal activity as revealed by inhibition of fungal growth around the wells containing the extracts while, growth of the fungal cultures was not inhibited around the control (Fig. 3). The visual observation suggests that the chloroform extract of both pH 7.0 and pH 4.5 did not have any significant difference in terms of inhibition zone formation against the test fungal pathogens except for the inhibition of *Fusarium verticilloides* by the extract obtained from the culture supernatant of pH 4.5.

Detection of biosynthetic genes involved in secondary metabolite production

The genes involved in the synthesis of secondary metabolites in *B. amyloliquefaciens* MBNC was detected by PCR amplification with gene specific primers. All the seven genes viz. *srfA* (surfactin biosynthetic gene) *dfnD* (gene for diffcidin biosynthesis), *fenA* (gene for fengycin biosynthesis), *baeR* (gene for bacillaene biosynthesis), *bacD* (gene for bacilysin biosynthesis), *dhbE* (gene for bacilibactin biosynthesis) and *mInA* (gene involved in biosynthesis of macrolactins) showed amplification. The amplified products of size ~900 bp for *dfnD*, ~890 bp for *srfA*, ~960 bp for *fenA*, ~ 980 bp for *ituA*, ~670 bp for *bacD*, ~ 940 bp *dhbE*, and ~ 920 bp for *mInA* confirmed the presence of genes responsible for antifungal activity (Fig.4).

Determination of bioactive antifungal compounds produced by *B. amyloliquefaciens* MBNC

The chloroform extract of *B. amyloliquefaciens* MBNC supernatant was further subjected to the High-Resolution Mass Spectroscopy (HR-MS) analysis to detect and identify the metabolites aiding the biocontrol ability. The HR-MS analysis revealed the presence of surfactins with varying chain length including surfactin C12 (exact molecular mass 993.6 g/mol, retention time 3.97), surfactin C13 (exact molecular mass 1007.65 g/mol, retention time 4.123) and surfactin C14 (exact molecular mass 1021.66 g/mol, retention time 4.43) were detected in the chloroform extract of both pH 7.0 and pH 4.5. Surfactin C15 (exact molecular mass 1035.68 g/mol, retention time 3.683) was exclusive to the extract of pH 4.5 with very low abundance, while production of surfactin C11 (exact molecular mass 979.60 g/mol, retention time 3.785) and iturin A (exact molecular mass 1042.54 g/mol, retention time 2.785) were only detected in pH 7.0 with low abundance (Fig.5).

Differential expression of *srfA* and *ituA* at pH 7.0 and pH 4.5 on Day 1 and Day 3

Quantitative real-time PCR analysis of *srfA* and *ituA* biosynthetic genes showed differential expression under acidic pH of 4.5. A significant difference in the expression of *srfA* at pH 4.5 was observed on day 1 but no significant difference was observed on day 3. However, *ituA* showed significant difference in its expression on both days at pH 4.5 (Fig.6). This indicated that pH of the medium had influence on the expression of the genes involved in surfactin and iturin biosynthesis. The similar expression pattern of *srfA* gene during day 3 in both acidic and neutral pH of bacterial culture confirmed the production of different surfactin derivatives in both the pH. However, the expression pattern of *ituA* gene did not correlate with the results of HR-MS analysis.

Effect of *B. amyloliquefaciens* MBNC on germination of seed of *Phaseolus vulgaris* infected with fungal phytopathogens

Bacillus amyloliquefaciens MBNC had a positive effect on the germination of seeds of bean (*Phaseolus vulgaris*) in terms of radicle formation, root elongation and suppression of fungal infection. The germination percentage and comparative root growth of bean under bacteria treated and untreated condition is shown in Fig. 7. The seeds that were pre-inoculated with bacterial suspension were able to counter the fungal phytopathogens and showed high germination efficiency. Although, there were no significant differences between bacteria treated and untreated seeds in terms of germination rate after 3 days of treatment (except for the seeds infected with *F. oxysporum*) (Fig. 7a), higher germination efficiency was observed after 7 days in bacteria treated seeds due to notable reduction in the fungal infestation by most of the fungal pathogens (Fig. 7b). Control (uninfected) seeds exhibited ~100% germination efficiency regardless of bacterial treatment. Bacterial pre-treatment also generated pronounced effect on the root growth after seed germination. There was remarkable growth in the primary root length of bacteria treated seeds compared to the bacteria untreated seeds during fungal infestation (Fig. 7c-e). The differences were more pronounced after 7 days of treatment and even control (uninfected) seeds after bacterial treatment exhibited longer primary root length compared to the untreated seeds, indicating the bacterial influence over seed germination. An exception to this finding was found in case of *Athelia rolfsii*, where infection could not be controlled even with bacterial pre-treatment of the seeds which had low germination efficiency regardless of the treatment with bacteria.

Plant growth promoting properties (PGP) of *B. amyloliquefaciens* MBNC:

The functional characteristics of *B. amyloliquefaciens* MBNC with regard to phytohormone (IAA and GA) production was evaluated both in neutral (pH 7.0) and acid stress (pH 4.5) condition. The pH of the medium had an influence on the functionality of the isolate. Secretion of Indole-3-acetic acid (IAA) and gibberellic acid (GA) by the bacterium under neutral condition was higher (12.82 ± 0.12 µg/ml and 35.15 ± 0.005 µg/ml respectively) than in acidic condition (7.27 ± 0.06 µg/ml and 26.66 ± 0.003 µg/ml) (Supplementary Fig. S2). The secretion of phytohormone in acidic pH reduced significantly ($P \leq 0.05$) compared to that of neutral pH.

Discussion

Several studies have reported the ability of *Bacillus* species to tolerate stress conditions such as high, acidity, alkalinity salinity or temperature (Niehaus et al. 1999; Horikoshi 2008). In this study, we took a previously isolated acid tolerant isolate of *B. amyloliquefaciens* MBNC with plant growth promoting ability to test for its

antagonistic activity against plant fungal pathogens in acid stress condition. *B. amyloliquefaciens* MBNC showed significant antagonistic activity against *P. myriotylum*, *F. incarnatum*, *F. redolens*, *F. verticilloides*, *F. oxysporum* and *S. rolfsii* in acid stress condition. The phytopathogenic fungi taken in this study are considered as significant threat to the agricultural crop production. The Genus *Pythium* comprises of several species that are mostly soilborne pathogens and cause serious economic loss on a wide variety by inhibiting seed germination, killing seedlings and reducing their vigour (Hendrix and Campbell 1973). *Fusarium* species belong to a large genus and cause wide range of plant diseases such as crown rot, head blight, and scab on cereal grains; vascular wilts in horticultural crops. Nearly all species produce mycotoxins that damage food and feed (Booth 1971). The fungus *Sclerotium rolfsii* infect plant parts by causing damping-off of seedlings, stem canker, crown blight, root, crown, bulb, tuber and fruit rots. The mycelia of *A. rolfsii* spread rapidly and can remain active in soil for long period as sclerotia (Billah et al. 2017). The management of fungal diseases through microbial biological control agents is a safer alternative to the use of agrochemicals and more sustainable method of agricultural production. The antifungal activity of *B. amyloliquefaciens* MBNC ascertained by dual culture disc diffusion method revealed maximum inhibition by 3 days grown culture supernatant compared to 7 days grown culture. Incubation period of the bacterial culture has an effect on the production of metabolites. *B. subtilis* produces active metabolites within the first 18 hours which remain stable until the 96th hour (Marajan et al. 2018). *B. amyloliquefaciens* is well recognized as biocontrol agent and plant-growth promoting bacteria (Bashan and Holguin 1998; Khan et al. 2018). The bacteria employ several strategies to act antagonistically against phytopathogens including secretion of lytic enzymes, production of antibiotics and volatile organic compounds and biosurfactants, (Haraguchi et al. 1999; Nunes 2012). Among the numerous antifungal compounds produced by *Bacillus* spp, lipopeptides belonging to the families of iturin, surfactin and fengycin are regarded as pivotal determinant of the biological control activity in *B. amyloliquefaciens* (Monteiro et al. 2016; Chen et al. 2020). These lipopeptides act on the fungal mycelial cell wall to inhibit conidial germination, causing morphological deformities and lysis of the mycelial cell wall leading to the death of the pathogen (Li et al. 2009; Kumar et al. 2012). Previous workers have successfully used strains of *B. amyloliquefaciens* as biocontrol agents to suppress several pathogens including *Pyricularia grisea*, *Sclerotinia sclerotiorum* and *Fusarium solani* (Yoshida et al. 2001; Ajilogba et al. 2013; Ji et al. 2013).

The organic fractions obtained from the chloroform extraction exhibited strong inhibition against the fungal isolates. An exception was observed in case of *Fusarium verticilloides* where the chloroform extract of the *B. amyloliquefaciens* grown in both pH conditions were less effective in inhibiting the growth of the fungus. The resistance of the pathogen may be attributed to the aggressive nature of the pathogen. *Fusarium verticillioide*s (synonym, *F. moniliforme*) with variable aggressiveness has been reported from many plant species including maize (Bacon et al. 1996; Leyva-Madriral et al. 2017). Chloroform extract of *B. amyloliquefaciens* have been reported to act against several fungal pathogens (Kadaikunnan et al. 2015). Butanol extract of the culture filtrate of *B. amyloliquefaciens* DA12 was reported to completely suppress the germination of *F. graminearum* macroconidia when used at a concentration of 250 µg/ml (Lee et al. 2017). The growth of the fungus was also strongly inhibited by the chloroform extracted organic fractions of *Bacillus subtilis* SCB-1 (Hazarika et al., 2019).

The genes viz., *srfA*, *dfnD*, *fenA*, *baeR*, *bacD*, *dhbE* and *mInA*, *srfA* and *ItuA* involved in synthesis of secondary metabolite in the genome of *B. amyloliquefaciens* MBNC were detected by PCR. The presence of secondary metabolite gene clusters in *Bacillus* encoding for number of polyketides (PKs) and lipopeptides (LPs) have

been reviewed (Jacques 2011; Aleti et al. 2015). A study on whole genome mining estimated that 31% of the Firmicutes harbor NRPS and PKS secondary metabolite gene clusters within their genome, of which majority (about 70%) encode NRPS while the rest encode PKS (Wang et al. 2014). The LPs are synthesized by NRPS gene clusters (Finking and Marahiel 2004). The secondary metabolites especially, PKs and LPs arm the bacteria with the ability to suppress disease in plants or activating plant immune response (Ongena and Jacques 2008; Zhang et al. 2016; Cochrane and Vederas 2016). Antifungal potential of *Bacillus subtilis* SCB-1 against fungal phytopathogens showed presence of five genes involved in the synthesis of bioactive polyketides (bacillaene, difficidin and macrolactins) and lipopeptide (surfactin and fengycin) (Hazarika et al. 2019). Bacillaene has been reported to show antagonistic activity against bacteria and fungi (Patel et al. 1995; Chen et al. 2018). Difficidin and macrolactins comprises a group of broad-spectrum antibiotics produced by *Bacillus* sp that functions by selective inhibition of protein synthesis (Schneider et al. 2007). Products of *surfA* and *fenA* are involved in the biosynthetic pathways of surfactin and fengycin, respectively (Cosmina et al. 1993; Hofemeister et al. 2004) and well known for their antifungal properties. Genome analysis of *Bacillus amyloliquefaciens* LL3 revealed the existence of genes encoding for the complete iturinA biosynthetic pathway (Gao et al. 2017; Dang et al. 2019). It exerts strong antifungal activity against an extensive range of plant pathogens (Leclère et al. 2005).

Further analysis to detect and determine the metabolites aiding in the biocontrol ability of *B. amyloliquefaciens* MBNC through HR-MS analysis revealed the presence of surfactins of different molecular weight and varying C-number (C11 – C15). The surfactin C12, C13 and C14 homologues were abundantly detected in both pH 7.0 and pH 4.5, suggesting the direct role of these three molecules in controlling the growth of fungal pathogens. Earlier studies suggested that production of surfactin is favoured by pH nearing neutral or varying towards slightly acidic range (Abdel-Mawgoud et al. 2008; Monteiro et al. 2016). An increase in the production level of surfactin and fengycin by a factor of 3.7 and 2.13 respectively on raising the pH level from 5.0 to 7.0 was reported earlier (Hmidet et al. 2017). Irrespective of the neutral or acidic pH, our findings suggested that surfactin variants were predominant in the culture supernatant of *B. amyloliquefaciens* MBNC. The antagonistic ability of our isolate to suppress fungal growth may be attributed to the production of surfactins in both pH conditions tested. The most commonly available surfactins in *Bacillus* species are surfactin C13, C14 and C15 (Liu et al. 2015; Plaza et al. 2015; Bartal et al. 2018). Recent studies reported the biosynthesis of surfactin C11 and C12 (Sarwar et al. 2018; Bartal et al. 2018). The antifungal activity of surfactin is due to its interaction with the cell membrane and disruption of membrane stability (Maget-Dana and Ptak 1995; Grau et al. 1999; Eeman et al. 2006). Iturins, fengycin etc are non-ribosomally synthesized lipopeptides that shows structural similarities to surfactin (Roongsawang et al. 2010). Previous studies reported that some *B. amyloliquefaciens* and *B. subtilis* strains could co-produce fengycin, iturin and surfactin (Athukorala et al. 2009; Arrebola et al. 2010). In our study, HRMS analysis also detected iturin A in the supernatant of pH 7.0. The findings indicate that the production of the lipopeptides viz. surfactins of varying C-chain length and iturin A by *B. amyloliquefaciens* is effective in the suppression of the fungal pathogens. The decreased antifungal activity of the bacterial supernatant of pH 4.5 against *F. verticillioides*, might be due to the absence of active iturin A in the chloroform extract of the bacterial culture grown in pH 4.5. Iturin A has been previously reported to govern the antifungal activity of *Bacillus amyloliquefaciens* DA12 against *F. verticillioides* and three other *Fusarium* species (Lee et al. 2017)

The results from HRMS analysis was further validated at transcript level through a qRT-PCR analysis to measure the expression level of *srfA* and *ituA* genes. The qRT-PCR analysis revealed increased expression of both the genes in Day 1 at pH 4.5. However, the transcript level of *ituA* on day 3 was higher at pH 4.5 while, the expression level of *srfA* did not vary significantly on day 3 at pH 4.5 compared to the neutral pH. Biosynthesis of lipopeptides in *Bacillus* is regulated by complex metabolic network (Shen et al. 2010). Specific pleiotropic regulators including, the quorum sensing cluster *comQXPA* regulates biosynthesis of surfactins. Abiotic factors including temperature and pH of culture medium influence the expression of genes involved in the production of subtilosin A and iturin A in *Bacillus* strains (Mizumoto and Shoda 2007; Velho et al. 2011). Despite, the abundance of *ituA* transcript level in both pH range, it was puzzling to note that HRMS analysis could not detect *ituA* at pH 4.5. This may be due to the regulatory steps leading to the final production of iturin A. Low pH of the medium affects the translation process of some genes (Katz and Orell 2012; Goswami et al. 2018). It is also possible that techniques employed in the present study to detect the iturin may not have been sensitive enough for which, more sensitive methods like Time of flight-secondary ion mass spectrometry (TOF-SIMS) may be required (Nihorimbere et al. 2012; Monteiro et al. 2016).

The efficiency of *B. amyloliquefaciens* MBNC in countering fungal infections during germination of bean seeds was established. Species of *Pythium*, *Fusarium* and *Sclerotium* are common seed-borne or soil-borne pathogens causing significant damages to common bean (Li et al. 2014; Mahadevakumar et al. 2015; Marcenaro and Valkonen 2016). Three major pathogens from the genus *Pythium* viz. *P. aphanidermatum*, *P. irregulare* and *P. myriotylum* are reported to cause damping off disease of bean (Binagwa et al. 2016). *Athelia rolfsii* (synonym. *Sclerotium rolfsii*) causes southern-blight with wilting and crown rot symptoms as well as leaf spot diseases of bean (Mahadevakumar et al. 2015). Likewise, several species of *Fusarium* cause wilt and root rot disease of bean, among of which *F. oxysporum*, *F. solani* and *F. incarnatum* are most common (Abdel-Razik et al. 2012; Marcenaro and Valkonen 2016). Experiment to evaluate the efficiency of *B. amyloliquefaciens* MBNC in suppressing fungal pathogens during the germination of bean seeds showed considerable efficacy against the test pathogens (except *A. rolfsii*). *B. amyloliquefaciens* MBNC could not counter the *A. rolfsii* infection due to its rapid and aggressive growth over the seed. Several researchers have previously reported the ability of *B. amyloliquefaciens* to inhibit pathogens in different vegetables including gray mold in tomato, and sclerotium rot in cucumber, powdery mildew in cucumber and pumpkin (Ji et al. 2013). The bacteria has also been used as biological control agents (BCA) to control green mold and blue mold rot on postharvest citrus (Yu 2009) and strawberry anthracnose (Essghaier et al. 2009). Iturin A produced by *B. amyloliquefaciens* effectively control *Rhizoctonia solani* have been reported earlier (Yu et al. 2002). In addition, Soybean seeds coated with *B. amyloliquifaciens* were able to colonize the seedling rhizosphere and successfully control *Rhizoctonia solani* (Yu and Sinclair 1996).

The influence of pH on the plant growth promotion ability of different bacteria has been reported earlier (Goswami et al. 2018). The secretion of phytohormone in acidic pH reduced significantly ($P \leq 0.05$) compared to that of neutral pH in this study. The adaptive mechanisms adopted by the bacteria under acid stress condition might have influenced the reduction in phytohormone secretion. Under acid stress condition, diversion of more energy to survival mechanisms rather than metabolic processes like production of IAA or GA is imperative. Moreover, activity of enzymes involved in biosynthetic pathway involving phytohormone production may have been influenced by the pH of the medium, leading to the reduction in production of the same under acid stress.

Conclusion

This study established the potential of the isolate *Bacillus amyloliquefaciens* MBNC as a promising candidate for biocontrol and plant growth promotion activities even under conditions of low pH. This isolate was found to be effective against several important fungal pathogens as it produces biometabolites such as surfactin and iturinA that aid in suppression of fungal phytopathogenic infections. Thus, this isolate is a promising biocontrol agent and can be effectively used against soil-borne fungal pathogen in acidic soil condition.

Declarations

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Tables

Table 1: Primers used in the study

Sl. No.	^a Primer name	Sequence (5'-3')	Annealing temperature (°C)	Product size (base pairs)	Description
1x	U16S-F	AGAGTTTGATCMTGGCTCAG	55	1500bp	encodes 16S rRNA gene
	U16S-R	TACGGYTACCTTGTTACGACTT			
2	ITS1-F	TCCGTAGGTGAACCTGCGG	50	550 to 650	encodes ribosomal Internal Transcribed Spacer (ITS) region
	ITS4-R	TCCTCCGCTTATTGATATGC			
3	BaeR-F	AGACTCCACCAAGGCAAATC	55	990	encodes gene for bacillaene biosynthesis
	BaeR-R	CAGCGGCTTCATGTCATACT			
4	DfnD-F	CAGGCGGAATAGGAGAAGTATG	54	900	encodes gene for difficidin biosynthesis
	DfnD-R	CGGCAGCCGATTGAAATAAC			
5	SrfA-F	GCTGATGATGAGGAGAGCTATG	55	890	encodes gene for surfactin biosynthesis
	SrfA-R	GATGGTCGATACGTCCGATAAA			
6	MlnA-F	GGCAGGGTCATACCTCATAATC	55	920	encodes gene for macrolactin biosynthesis
	MlnA-R	AGCAGACTTTCGGTCTCATTC			
7	DhbE-F	GCTGGAGGAAGAGTGGTATTATC	54	940	encodes gene for bacilibactin biosynthesis
	DhbE-R	CAGTAAATGAAGCGGCGTTATG			
8	BacD-F	CCGGCGTCAAGTCTATCAAA	54	670	encodes gene for bacilysin biosynthesis
	BacD-R	CATGGCTCCTGCTCCAATAA			
9	ItuA-F	CGGGAAACAACAGGCAAATC	55	980	encodes gene for iturin biosynthesis
	ItuA-R	CGTCACCAGCGGTGTAAATA			
10	FenA-F	CATTCATCCTGGAGACCCTATTC	55	960	encodes gene for fengycin biosynthesis
	FenA-R	TAAGACCGCAGGCATGTTATAG			
11.	qSrfA	CGTTAGCGGGGATGAAACTG	58.99		encodes gene for surfactin biosynthesis (used for qPCR analysis)
	qSrfA	GATGCTCTCAGCCGCCTTC	60.89		
12.	qltuA	GAGGCAACTCCATCCGAGC	59.28		encodes gene for iturin biosynthesis (used for qPCR analysis)
	qltuA	GCATTTCTTTGCAGCCGGA	59.75		
13.	q16S-F	GTGTCGTGAGATGTTGGGTTA	64		16S (encodes 16S rRNA gene)
	q16S-R	GTGTGTAGCCCAGGTCATAAG	64		

Table 2. Results of BLAST analysis of the ITS sequences of the isolates

Isolate Code	ITS Identification	Identity (%)	Accession number
1NC	<i>Pythium myriotylum</i>	99.64	MT367536
2NC	<i>Fusarium verticilloides</i>	99.45	MT367537
3NC	<i>Fusarium incarnatum</i>	100.00	MT367538
4NC	<i>Athelia rolfsii</i>	99.03	MT367539
5NC	<i>Fusarium oxysporum</i>	99.21	MT367540
6NC	<i>Fusarium redolens</i>	98.84	MT367541

Table 3: Inhibition percentage (%) of the fungal phytopathogens by the supernatant of 3 days and 7 days old culture of *B. amyloliquefaciens* MBNC

^a Fungal Isolates	Inhibition percentage (%)		Significant (p≤0.01)	Inhibition percentage (%)		Significant (p≤0.01)
	Bacterial supernatant from 3days old culture	Bacterial supernatant from 3days old culture		Bacterial supernatant from 7days old culture	Bacterial supernatant from 7days old culture	
	pH 7.0	pH 4.5		pH 7.0	pH 4.5	
<i>Pythium myriotylum</i> 1NC	50.1±0.49	60.03±0.61	Yes	20.83±0.49	14.54±0.35	Yes
<i>Fusarium incarnatum</i> 3NC	28.7±0.43	36.39±0.35	Yes	23.67±0.43	5.40±0.22	Yes
<i>Fusarium redolens</i> 6NC	54.11±0.64	63.53±0.29	Yes	50.13±0.61	9.97±0.38	Yes
<i>Fusarium verticilloides</i> 2NC	25.13±0.52	9.77±0.38	Yes	27.67±0.46	19.84±0.25	Yes
<i>Fusarium oxysporum</i> 5NC	47.17±0.55	60.07±0.52	Yes	41.25±0.49	11.11±0.43	Yes
<i>Athelia rolfsii</i> 4NC	25.43±0.38	34.93±0.46	Yes	1.16±0.03	3.02±0.14	Yes

^a The data shown in the table are the average±standard error (n=3). The data were analysed for significant difference using student *t*-Test (Paired two sample for means) and $p\leq0.01$ were considered as significant.

Table 4. Germination percentage of tomato seeds upon (a) treatment with fungal phytopathogens verses (b) pre-treatment with *B. amyloliquefaciens* MBNC prior to treatment with fungal phytopathogens

Fungal Isolates	^a Seeds treated with only fungus (%)	Seeds treated with MBNC and fungus (%)
<i>Pythium myriotylum</i> 1NC	25.00±4.81	68.44±3.39
<i>Fusarium incarnatum</i> 3NC	36.11±2.78	75.00±4.81
<i>Fusarium redolens</i> 6NC	36.11±2.78	77.77±5.56
<i>Fusarium verticilloides</i> 2NC	18.89±3.09	44.44±2.78
<i>Fusarium oxysporum</i> 5NC	33.33±4.81	77.78±2.78
<i>Athelia rolfsii</i> 4NC	13.00±2.46	25.00±0.88

^a The data shown in the table are the average±standard error (n=3). The data were analysed for significant difference using student *t*-Test (Paired two sample for means) and $p\leq0.01$ were considered as significant.

Figures

Figure 1

Neighbor joining (NJ) tree of the fungal isolates (Isolates with bullet is considered in the study while the rest were considered from NCBI database). The phylogenetic tree, constructed using the ITS sequences of the fungal pathogens, revealed their close evolutionary relationships with the type species.

Figure 2

Antagonistic activity of *B. amyloliquefaciens* MBNC against fungal phytopathogens at pH 4.5 and pH 7.0.

Figure 3

Well diffusion assay for detection of antifungal activity of chloroform extract of *B. amyloliquefaciens* MBNC at pH 4.5 and pH 7.0 against (a) *Pythium myriotylum* 1NC, (b) *Fusarium incarnatum* 3NC, (c) *F. redolens* 6NC (d) *F. verticilloides* 2NC (e) *F. oxysporum* 5NC and (f) *Athelia rolfsii* 4NC (7 indicates extract from pH 7.0, 4.5 indicates extract from pH 4.5, C indicates control wells with DMSO)

Figure 4

PCR amplified products of the eight secondary metabolite biosynthetic genes of *B. amyloliquefaciens* MBNC on 1.2% agarose gel.

Figure 5

Detection of bioactive metabolites produced by *B. amyloliquefaciens* MBNC using HRMS (a) Total Ion Chromatogram (TIC) of *B. amyloliquefaciens* MBNC extract grown at pH 7.0. (b) TIC of *B. amyloliquefaciens* MBNC extract grown at pH 4.5. (c) MS spectra of specific compounds detected in the TIC of extract collected from pH 7.0. (d) MS spectra of specific compounds detected in the TIC of extract collected from pH 4.5. The numbers mentioned in (a) and (b) denotes the compounds (i) iturin A (ii) surfactin C15 (iii) surfactin C11 (iv) surfactin C12 (v) surfactin C13 (vi) surfactin C14. The retention time for each compound is mentioned along with the spectra.

Figure 6

Differential gene expression of *srfA* and *ituA* at pH 7.0 and pH 4.5 on Day 1 and Day 3 by quantitative real-time PCR.

Figure 7

Biocontrol efficiency of *B. amyloliquefaciens* MBNC against fungal infestation in germinating seeds of bean (*Phaseolus vulgaris*). (a) Germination percentage of bacteria treated and untreated seeds after 3 days of germination under fungal infestation, (b) Germination percentage of bacteria treated and untreated seeds after 7 days of germination under fungal infestation, (c) Average root length of germinated seeds after 3 days of germination, (d) Average root length of germinated seeds after 7 days of germination, (e) Photographic evidence showing the differences in the root length of bacteria treated and untreated seeds after 7 days of germination.

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