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Antifungal Activity of Lipopeptides from *Bacillus subtilis* Isolates Against Rhizome Rot of Ginger Caused by *Fusarium oxysporum* and *Pythium aphanidermatum*

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Abstract

Research for the development of biological control agents as an alternative to agrochemicals for controlling plant pathogens has gained considerable attention for the acquirement of agricultural sustainability. *Fusarium* is a complex genus of ascomycete fungi that consists of plant pathogens of agricultural relevance. The genus *Pythium* is one of the most important groups of soil-borne plant pathogens, present in almost every agricultural soil and attacking the roots of thousands of hosts, reducing crop yield and quality. In the present study, an analysis of the bio-control attributes of *Bacillus subtilis* has been conducted and demonstrated the most effective antagonism against *Fusarium* spp. and *Pythium* spp. under *in vitro* conditions. Bacteria, especially members belonging to the genus *Bacillus* are well recognized for defending the plants against various phytopathogens due to their ability to produce a variety of secondary metabolites and siderophores. *Iturin A* gene was PCR amplified and showed 675bp length. The lipopeptide was extracted and quantified ($O.D._{280}$) from two strains T_3 (35µg/ml) and T_6 (18µg/ml). The extracted lipopeptide and cell-free extract (supernatant) of T_3 and T_6 strains of *Bacillus subtilis* were used at a concentration of 25µg/ml and 10µl/ml of media was used against *Fusarium oxysporum* and *Pythium aphanidermatum,* respectively to check the efficacy of antifungal activity. The antifungal activity of the bacterial isolates against pathogens was recorded and a growth inhibition zone was observed. It was observed that 4 cm radius in control without inhibition. The poison food technique method with lipopeptide of T_3 and T_6 strains showed 36.79 per cent and 51.46 per cent inhibition activity and cell-free extract of T_3 and T_6 strain showed 33.54 per cent and 52.63 per cent inhibition activity, respectively against *Fusarium oxysporum*. Also, lipopeptide of T_3 and T_6 strains showed 5.33 per cent and 11.46 per cent and cell-free extract showed 1.46 per cent and 1.25 per cent inhibition activity respectively against *Pythium* spp.

Keywords : Lipopeptides, *Bacillus subtilis*, *Fusarium oxysporum* and *Pythium aphanidermatum*

Soil bacteria are the most abundant and diverse group of microorganisms on earth and several species have been widely utilized as potent bio-inoculants for improving plant growth and disease management (Ambrosini *et al*., 2016). Global plant disease outbreaks are increasing and threatening food security. Crop protection is pivotal to maintaining abundant production of high quality crops. Over the

past 100 years, the use of chemical fertilizers and pesticides and good agronomical practices enabled growers to maintain improved crop productivity. However, extensive use of chemicals during the last few decades in controlling pests and diseases resulted in negative impacts on the environment, producing inferior quality and harming consumer health. In recent times, diverse approaches are being used to manage and / or mitigate a variety of pathogens for the control of plant diseases. Biological control is the alternative approach for disease management that is eco-friendly and reduces the amount of human contact with harmful chemicals and their residues. A variety of biocontrol agents including fungi and bacteria have been identified but require effective adoption and further development of such agents. This requires a better understanding of the intricate interactions among the pathogen, plants and environment towards sustainable agriculture. Beyond the field assessment, the analysis of microbial communities with culture-independent molecular techniques including sequencing technologies and genomics information has begun a new era of plant disease management. Western Ghats of India are one of the thirty-fourth biodiversity hot spots in the world. The biodiversity of Western Ghats has been richly explored in the form of flora and fauna but microbial forms have not been fully evaluated. The present study has been undertaken to isolate and characterize *Bacillus subtilis*, a major antibiotic-producing filamentous bacterium from Western Ghats soils of Karnataka, India. The use of biological control agents for the management of plant pathogens is considered a safer and sustainable strategy for safe and profitable agricultural productivity. *Bacillus* based biocontrol agents play a fundamental role in the field of biopesticides. Many Bacillus species have proved to be effective against a broad range of plant pathogens. They have been reported as plant growth promoters, systemic resistance inducers and used for the production of a broad range of antimicrobial compounds (lipopeptides, antibiotics and enzymes) and competitors for growth factors (space and nutrients) with other pathogenic microorganisms through colonization. (Kumbar *et al*., 2021; Ritapa Datta & Ramesh, 2022 and Ganavi & Ramesh, 2022). Among the members of the *Bacillus* genus, *Bacillus subtilis* strains have special potential to be bio-active and eco-friendly agents for controlling diseases. In addition, *Bacillus subtilis* strains, treated with gold, aluminium and silver-coated nanoparticles have been reported not only to significantly increase the plant growth but also to inhibit the growth of harmful fungal parasites within the rhizosphere, thus acting as potential nano-biofertilizers (Gouda *et al*., 2018). Their biocontrol effects can be attributed to their ability to induce host-plants natural defence response mechanisms to a wide range of pathogens (Lastochkina *et al*., 2018) and their generally recognized status as safe microorganisms to use in the food industry (Arroyave *et al*., 2017). Furthermore, *Bacillus subtilis* strains are easily formulated and stored due to their ability to produce endospores that are resistant to dynamic physical and chemical treatments, such as heat, desiccation, organic solvents and UV irradiation, and therefore maintain their ability to trigger defence responses in host plants, even under unfavourable conditions (Gao *et al*., 2016). Iturins and closely related lipopeptides constitute a family of antifungal compounds known as iturinic lipopeptides that are produced by species in the *Bacillus subtilis* group. Iturins are an important class of lipopeptides that have been widely studied for their antibiotic activities and are produced by members of the *Bacillus* group (Ongena and Jacques, 2008). Iturins was first reported as an antibiotic produced by *Bacillus subtilis* in 1950 and named after the Iturin region in the Congo where the strain was isolated (Delcambe, 1950). The chemical structure of iturin A was later reported to be a cyclic heptapeptide with an alkyl chain (Peypoux *et al.,* 1978). The mode of action of these lipopeptides was shown to be pore formation in cell membranes (Besson *et al*., 1984 and Maget-Dana *et al*., 1985). All of them have been shown to have strong antifungal activity and they are known active ingredients in many biological control products that target fungal plant pathogens (Ongena and Jacques, 2008). In addition to their antifungal activity, they have been shown to induce defence responses in plants. (Farace *et al.,* 2015; Park *et al*., 2016 and Wu *et al.,* 2018). The aim of this study was to characterize

antifungal lipopeptides and to check the efficacy of strains of *Bacillus subtilis* against *Fusarium oxysporum* and *Pythium aphanidermatum.*

Material and Methods

Exploration for *Bacillus* spp. from different soil environments exhibiting multiple plant-growth-promoting and biocontrol traits have attracted considerable attention from researchers in recent years.

Growth Conditions of Bacterial Strains

All the *Bacillus subtilis* strains were grown at a 30^0C incubator with shaking at 180 - 200 rpm using NA and LB broths or NA, LB and BDA plates. Also, all the *Bacillus subtilis* strains were maintained as glycerol stock, freshly *Bacillus subtilis* strains grown for 24 hours in LB medium and the final concentration of 20 per cent sterile glycerol was added, transferred to cryovials and stored at -80^0C. Glycerol stocks were made as described for *Bacillus subtilis* and stored at -80^0C.

Table 1

Bacillus subtilis strains used in this study

Bacterial strain name	Strain id
Bacillus subtilis	MTCC (T3)
Bacillus subtilis	BJP3 (T6)

Source : (1) The Microbial Type Culture Collection and Gene Bank (MTCC)
(2) Western Ghats of Karnataka (BJP3)

Fungal Pathogen

The pure culture of *Fusarium oxysporum* and *Pythium aphanidermatum* which was isolated from ginger causing rhizome rot disease and then grown on PDA media at 28ºC in a BOD incubator. The entire Petri plate was covered within 5 days with the optimum growth conditions.

Collection of Soil Samples

Soil samples were collected from Western Ghats of Karnataka randomly for isolating *Bacillus subtilis*. 5-10 gram of soil sample was collected from 5 cm depth, after gently removing the debris on the top layer of soil, using a sterile spatula and placed immediately inside the sterile polythene cover. Labels containing the details on date of collection, place of collection, collector's name, description of the place of collection, and the agro-climatic zone in which the sampling was carried out, labels were placed inside the bag. The soil samples were stored at 4^0C for further work (Kumbar *et al*., 2017 and Kumbar *et al*., 2019).

Isolation of *Bacillus subtilis* Strain

In a microfuge tube, suspended a small amount (a loop full) of soil in 1 or 2 drops of distilled water. Mixed well and incubated at 80°C heating block for 10 minutes, to eliminate most of the gram-positive and gram-negative bacteria. After cooling the heat-treated soil samples were streaked onto nutrient agar plates using an inoculation loop. Incubated at 30°C for 1-2 days and after incubation different colonies were observed onto nutrient agar media. White and dry or pasty looking colonies were picked up and re-streaked onto Bacillus differentiation Agar media and incubated at 30°C for 1-2 days. All the yellow colonies observed in Bacillus differentiation agar are taken for isolation and streaked on to the master plate. Strains used in the study are T3 strain as reference strain from MTCC (Microbial Type Culture Collection, Chandigarh) and T6 strain (BJP 3) isolated from forest soils of Western Ghats of Karnataka.

Maintenance of Strains

Bacillus colonies were inoculated into 100 ml of LB broth and incubated at 30°C at 180-200rpm. Simultaneously all the colonies were examined for releasing of spore. 1ml of overnight grown cultures were transferred to the cryogenic vial, 50 and 60 per cent glycerol was added, vortexed and stored at -80°C.

PCR Amplification of Strains

Bacterial genomic DNA was used to amplify the *Iturin A* gene. 1.25 U/ìl of *Taq* DNA polymerase (Thermo Scientific, 5U/µl), 2mM dNTPs each, 1µM both primers and 3mM of $MgCl_2$ to the final volume 20µl. Amplification was carried out using an Eppendorf thermocycler. Following parameters were used for amplification, 2 min of initial denaturation at 95°C followed by 30 cycles of amplification with a 40

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TABLE 2

Iturin A primers nucleotide sequence

Primer name	Primer sequence
IT Kpn For	GCCGGTACCATGAAAATTTAC GGAGTATATATGGA
IT Sal Rev	CCGTCGACTTATAACAGCTCTTC ATACGTTTTCAT

seconds denaturation at 95°C, 45 seconds of annealing at 51°C, and 1 min of extension at 72°C. An extra one final extension step of 15 min at 72°C was added after completion of the 30 cycles

Gel Electrophoresis

After the completion of the PCR amplification, 10-15µl of amplified products were used to check the amplification in 1 per cent agarose gel casting with ethidium bromide staining in TAE buffer, at about 80 volts until the marker dye reached near to the end of the gel. Gels were photographed under UV transilluminator.

***In-vitro* Bioassay**

Fungal Culture Maintenance

Purecultures of *Fusarium oxysporum* and *Pythium aphanidermatum* pathogen was isolated from ginger was maintained by subculturing on PDA (Potato Dextrose Agar) media and incubated at 28ºC for 5 days where the hyphal growth covered the entire petri plate and then stored at 4°C for further use.

Pure Bacterial Culture Maintenance

Colonies of *Bacillus subtilis* which are gram positive and spore forming were re-streaked on Bacillus differentiation agar (BDA Agar) and incubated at 30°C for 1-2 days. Confirming the growth morphology culture plates were maintained at 4°C with frequent sub culturing.

Lipopeptide Extraction

Preparation of Starter Culture and Main Culture

Starter culture of T_3 and T_6 strains of *Bacillus subtilis* was prepared by inoculating loop full of pure culture of *Bacillus subtilis* from Petri plate into the 100 ml of LB broth media prepared along with the control. Then it was kept for shaking in incubator cum shaker overnight at 30ºC and 150 rpm. The main culture was prepared from starter culture by inoculating 15 ml of each strain for 1.5 litre of LB Broth (added glycerol 15ml/1.5ltr). Then it was kept for shaking in incubator cum shaker for 4 days at 30ºC and 150 rpm.

Centrifugation and Acid Precipitation

Supernatant *i.e.,* cell free extract was collected from main culture after centrifugation in centrifuge at 7500rpm for 20 min. Acid precipitation was carried out by adding 2N concentrated HCl to the supernatant to lower the pH to 2 from 9. Then it was left overnight for complete precipitation at 4°C.

Separation of Lipopeptide Layer

The supernatant which was acid precipitated was mixed with chloroform and methanol in 2:1 ratio and stirred in magnetic stirrer for 15 min. The mixture was added into separating funnel and left for 4 hours for separation of layers. Then the middle layer was collected which was white coloured and semi-liquid in appearance. The left out upper and lower layer was again reextracted thrice in the same way for collection of remaining lipopeptides. Then the layer was collected and dissolved in equal quantity of methanol and then it was syringe filtered and stored at -20ºC for further use.(Cao *et al*., 2012).

Antifungal Activity by Poison Food Technique Method

The PDA media was prepared by autoclaving and after cooling, it was mixed with supernatant 10µl/ml media with each of T3 and T6 separately and also lipopeptide of 25µg/1ml (T3) and 25µg/1ml (T6) of media separately and allowed to solidify. The 25ml of media was poured into each petri plate and then 6mm disc of *Fusarium oxysporum* was placed at the exact centre. The PDA plate inoculated with fungal disc at the centre and without mixing supernatant or lipopeptide was used as control, Further PDA plates per treatment were replicated thrice. All these plates were incubated at 27± 2ºC in incubator. Observations on radial

mycelial growth of the fungal pathogens was measured and per cent inhibition was calculated (Leelasuphakul *et al.,* 2008). Inhibition rate of pathogen (IR) was calculated using the formula as follows(Melkamu *et al*., 2013).

$$IR = [(C2-C1)/C2] \times 100$$

Where, C2 is the control colony radius and C1 is the average radial growth of pathogen in the presence of antagonist.

Results and Discussion

PCR Screening of *B. subtilis* with Gene Specific Iturin A Primers

To identify the Iturin A gene, genomic DNA extracted from *B. subtilis* isolates was used as a template in PCR reactions and Iturin A gene-specific primers with *Kpn* I and *Sal* I restriction sites, respectively are used. Then the PCR products were amplified obtained at 675bp. (Fig 1).

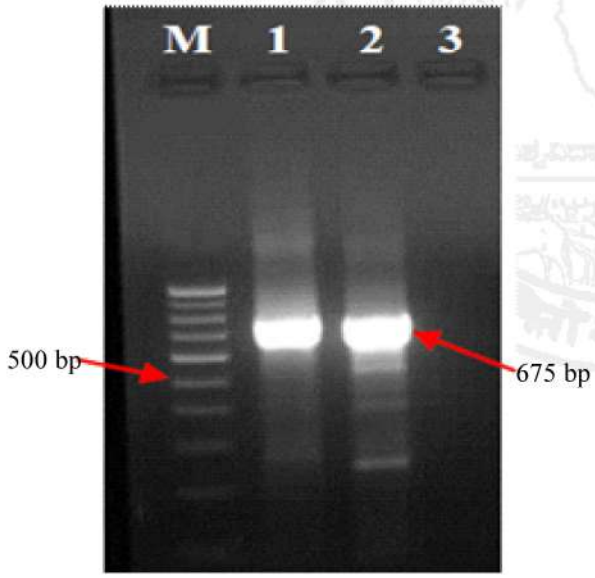

Fig. 1 : Agarose (1.0%) gel electrophoresis of detection of Iturin A gene of *Bacillus subtilis* by using Iturin A specific primers (A: M- 100bp Molecular weight marker, Lane 1 and 2: T3 and T6 strain and Lane 3: Genomic negative)

Antifungal Activity by Poison Food Technique Method

The *Bacillus* strains T3 and T4 were isolated, observed for morphological characters and confirmed based on morphology. The lipopeptide extraction was done by preparing starter culture and the main culture was prepared, then it was centrifuged to take out the supernatant and it was acid precipitated. On the next day, the supernatant was subjected to solvent extraction method and the lipopeptide layer was collected. The lipopeptide was dissolved in methanol and filter sterilized. The quantification of lipopeptides from strains was done with a spectrometer ($O.D._{280}$) and the values obtained for T3 and T6 was 35µg/ml and 18µg/ml, respectively. The extracted lipopeptide and cell-free extract (supernatant) of T3 and T6 strains of *Bacillus subtilis* were used at a concentration of 25µg/ml and 10µl/ml of media was used against *Fusarium oxysporum* and *Pythium aphanidermatum,* respectively to check the efficacy of antifungal activity. The antifungal activity of the bacterial isolates with lipopeptides and cell-free extract against *Fusarium oxysporum* and *Pythium aphanidermatum* was recorded and a growth inhibition zone was observed (Fig. 1). The observation was taken for *Fusarium oxysporum* and *Pythium aphanidermatum,* it was found that 4cm radius in control with 0 per cent inhibition rate. According to the poison food technique method lipopeptide of T3 and T6 strain showed 36.79 percentage and 51.46 percentage (Fig. 2) inhibition activity, cell-free extract of T3 and T6 strain showed 33.54 percentage and 52.63 percentage (Fig. 3). inhibition activity against *Fusarium oxysporum*. (Table 3). Observations were analysed statistically by SAS software and the graph depicting the inhibition showing antifungal activity was obtained (Fig. 4). Also, lipopeptide of T_3 and T_6

Fig. 2: Antifungal activity of lipopeptides of *Bacillus subtilis* against *Fusarium oxysporum,* (a) Control (b) R1, R2, R3 Replications of T3 strain (c) R1,R2,R3 Replications of T6 strain

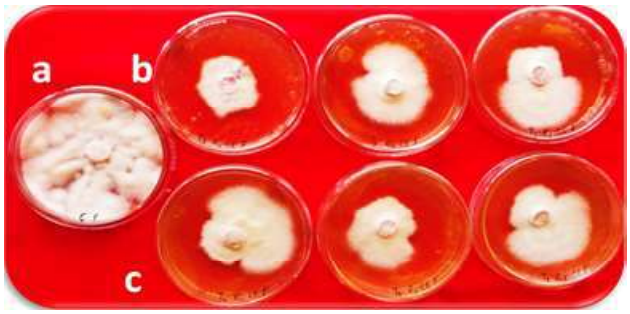

Fig 3: Antifungal activity of cell free extract of *Bacillus subtilis* against *Fusarium oxysporum* (a) Control (b) R1, R2, R3 Replications of T3 strain (c) R1,R2,R3 Replications of T6 strain

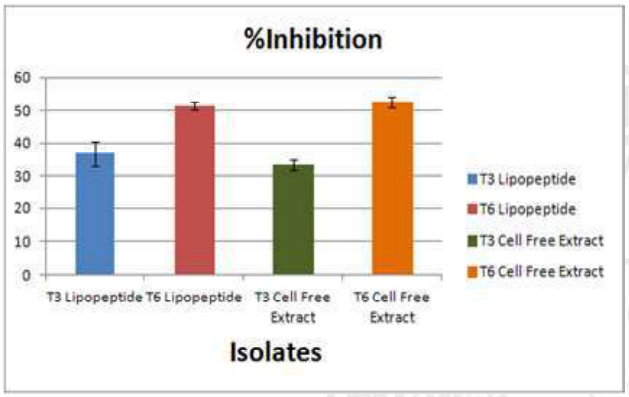

Fig. 4: Antifungal activity of lipopeptide and cell free extract of T3 and T6 strains of *Bacillus subtilis* against *Fusarium oxysporum*

strains showed (25.33%) and (11.46%) (Fig. 5) and cell-free extract showed (1.46%) and (1.25%) (Fig. 6) inhibition activity respectively against *Pythium aphanidermatum* . (Table 4, Fig. 7). Both lipopeptide and cell-free extract of T3 and T6 strain was effective against *Fusarium oxysporum* as compared to *Pythium aphanidermatum*.

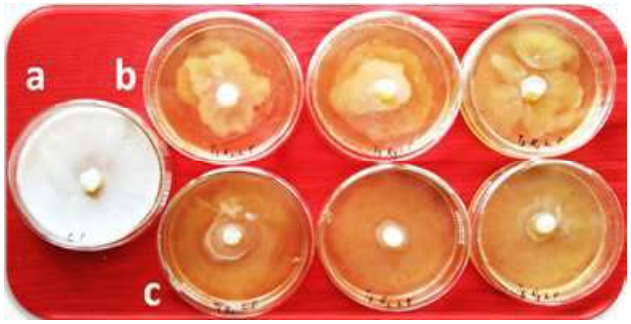

Fig. 5: Antifungal activity of lipopeptides of *Bacillus subtilis* against *Pythium aphanidermatum,* (a) Control, (b) R1, R2, R3 replications of T3 strain (c) R1,R2,R3 replications of T6 strain.

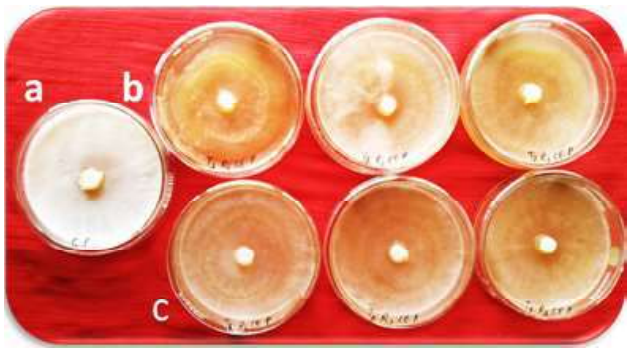

Fig. 6 : Antifungal activity of cell free extract of *Bacillus subtilis* against *Pythium aphanidermatum,* (a) control, (b) R1, R2, R3 replications of T3 strain (c) R1,R2,R3 replications of T6 strain.

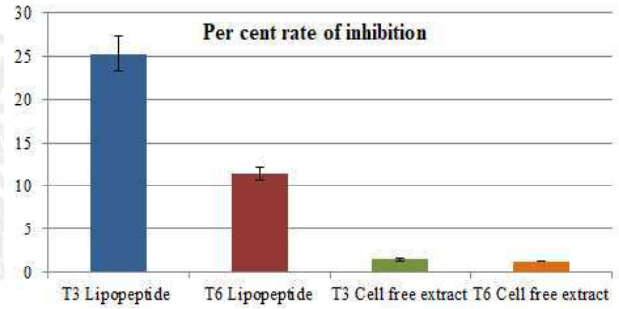

Fig. 7: Antifungal activity of lipopeptide and cell free extract of T3 and T6 strains of *Bacillus subtilis* against *Pythium aphanidermatum*

TABLE 3

Percent inhibition of lipopeptide and cell free extract of T3 and T6 strain of *Bacillus subtilis* against *Fusarium oxysporum*

Strains	Isolates	% Inhibition
T_3	Lipopeptide	36.79 ± 3.58 [b]
T_6	Lipopeptide	51.46 ± 1.27 [a]
T_3	Cell Free Extract	33.54 ± 1.63 [b]
T_6	Cell Free Extract	52.63 ± 1.66 [a]

TABLE 4

Percent inhibition of lipopeptide and cell free extract of T3 and T6 strain of *Bacillus subtilis* against *Pythium aphanidermatum*

Strains	Isolates	% Inhibition
T_3	Lipopeptide	25.33 ± 2.04 [a]
T_6	Lipopeptide	11.46 ± 0.76 [b]
T_3	Cell Free Extract	1.46 ± 0.21 [c]
T_6	Cell Free Extract	1.25 ± 0 [c]

Therefore, understanding the biocontrol mechanisms of *B. subtilis* will help us to evaluate and improve the biological control crop diseases. Biological control of soil-borne plant pathogens is associated to the synthesis of antibiotics and hydolytic enzymes, considered as traits defining biocontrol ability. Antibiosis through the production of antifungal metabolites and antibiotics is probably the best known and most important mechanism used by biocontrol bacteria to limit pathogen invasion in host plant tissues (Khedher *et al.,* 2021). In recent years, the need for biological control of plant pathogens has significantly increased as sustainable and environmentally friendly alternatives to the massive use of fungicides (Ongena and Jacques, 2008). Bacteria, especially members belonging to the genus *Bacillus* are well recognized for defending the plants against various phytopathogens due to their ability to produce a variety of secondary metabolites. Lipopeptides are surface-active compounds having both amphiphilic and biological properties and are known as biosurfactants (Arrebola *et al*., 2010) include bacteriocins, antimicrobial peptides, lipopeptides, polyketides and siderophores (Caulier *et al*., 2019). The majority of the studies carried out so far have focused only on the biocontrol potential of this bacterium to reduce fusarium diseases. In this back ground, the present study reports the biological potential of *Bacillus subtilis* strains to control rhizome rot of ginger caused by *Fusarium oxysporum* and *Pythium aphanidermatum*. Isolation of multitrait bacteria, *i.e.,* having antagonistic as well as growth promotion properties might be used as one input that promotes the growth and manages the disease thereby reducing chemical (inorganic fertilizers and fungicides) inputs in agriculture (Sukanya *et al*., 2018; Cao *et al.,* 2018). In the present study inhibition of *Fusarium* and *Pythium* by the lipopeptide and cell free extract of *Bacillus subtilis* strains was observed. The T3 and T6 strain showed the inhibition against *Fusarium oxysporum* as reported by Khedher *et al*., 2021 and Yao *et al*., 2003. The mechanism of direct antagonism shown by the members of *Bacillus* is attributed to the production of toxic secondary metabolites and lytic enzymes (Ji *et al*., 2013). Inhibition observed due to Bacillus isolates against Fusarium where the inhibition observed from the isolated strain (T6) and the inhibition was also observed from the reference strain (T3). In the same way, strong antagonistic activity was observed when lipopeptide was used against anthracnose of chilli. (Kumar *et al*., 2021). The role of *Bacillus* in promoting plant growth and defending the plant against various pathogenic agents is described in the literature (Cao *et al.,* 2018; Sukanya *et al.*, 2018).The current research revealed that strains considered for the study was effective in inhibiting the pathogens showing the strong antifungal activity. The *in vitro* results showed that *Bacillus subtilis* isolates such as lipopeptide and cell free extract have a great potential to be used as biocontrol agent for management of pathogens like *Fusarium* as compared to *Pythium* and helps in reducing crop loss due to pathogens and also maintains good soil health, promotes plant growth and development. Microbial lipopeptide has emerged as a new tool to overcome the future problems of hazardous chemical pesticides, with the safe and biodegradable lipopeptide like biopesticides, which are molecules act as biocontrol agents against the phytopathogens. Lipopeptides are made a special class of bioactive secondary metabolites have potential applications in the field of medical, agriculture, Phytosanitation. Due to the various applications of the lipopeptides, these molecules of low molecular mass are considered as versatile weapons.

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