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SHORT COMMUNICATION

Bacterial antagonists against *Ganoderma lucidum* the incitant of root rot of Indian Mesquite

Ritu Mawar[1] · Ladhu Ram[2] · Deepesh Sharma[1] · Kamlesh Jangid[3]

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Abstract

Indian arid regions recently witnessed large scale mortality in the Indian mesquite {*Prosopis cineraria* Druce}, locally known as Khejri tree, due to root rot caused by *Ganoderma lucidum*. Use of biological control agents offers an attractive alternative to manage *Ganoderma* induced diseases in Khejri tree, without any negative impact on the environment. Therefore, efforts were made to isolate antagonistic bacterial isolates from infected trees of *P. cineraria*. Several soil samples were analysed and two bacterial isolates: strains AZAC-1and AZ-11were selected as potential antagonists against *G.lucidum* from arid soils. Taxonomic identity of the two strains was ascertained using 16S rRNA in EzBioCloud. The strains showed over 99.7% similarity and were identified as *Streptomyces* sp.strain AZAC-1 (MK459414) and *Bacillus* sp.strain AZ-11(MH304296).

Keywords *Ganoderma lucidum* · *Streptomyces* sp. · *Bacillus* sp. · *Prosopis cineraria* · 16S rRNA gene · Antagonists

Ganoderma lucidum causes root rot of many tree species as well as shrubs in Indian arid and semi-arid regions (Lodha et al. 1986; Lodha and Harsh 2009). The disease is mostly prevalent in sandy soils where trees are raised under rainfed conditions. During recent years, arid regions witnessed large scale mortality of Indian mesquite {*Prosopis cineraria*}due to root rot caused by *G. lucidum* (Mawar et al. 2020).The tree, locally known as Khejri, is the most important component of agroforestry systems prevalent in the region. Recommended management strategies were effective only in partial control of the disease (Ahmed et al. 2004; Lodha and Harsh 2009). The use of biological control agents (BCAs) offers an attractive alternative for the management of *Ganoderma* induced diseases, without any negative impact on the environment. Potential antagonistic BCAs have showed efficacy in controlling other species of *Ganoderma.* For instance, controlling *G. boninense* colonization and infection in both nursery and field conditions (Suryanto et al. 2012; Biviet al. 2010) have been reported. This serves as a proof of concept that the disease can be kept under control by biological means. However, in arid regions the temperature of bare soil often reaches 50–55 °C during summer months. Under these conditions, BCAs which can survive and withstand such adverse conditions are likely to be more effective and useful. In this study, efforts were made to explore the possibility of heat tolerant native bacterial BCAs from the arid soils to manage root rot incited by *G. lucidum*.

All the laboratory experiments were conducted at ICAR-Central Arid Zone Research Institute, Jodhpur, India. The texture of soil used in the present study was sandy loamy with 85% sand, 8.8% clay and5.5% silt. The nutrient profile of soil included nitrogen (0.031%), organic carbon (0.25%) and 7 µg g^{-1} Olsen P with a pH of 8.1 and a moisture holding capacity (MHC) of 10.4% (w/w).Basidiocarps and infected roots were collected from Khejri trees, washed thoroughly with sterile water and then infected roots were cut into small pieces (0.5–1 cm) and surface sterilized (0.1% mercuric chloride) for 30 s before washing again in sterile distilled water. Fragments were incubated in sterilized bags along with wet cotton at room temperature for about 8–10 days. When mycelial growth on these pieces was observed it was transferred into Potato Dextrose Agar (PDA) medium and the inoculated Petri dishes were incubated at 28 ± 1 °C for 3–5 days before sub-culture onto PDA slants. Pure culture of

✉ Ritu Mawar
ritumawar1976@gmail.com

[1] ICAR-Central Arid Zone Research Institute, Jodhpur 342003, India

[2] College of Horticulture and Forestry, Jhalarapatan, Jhalawar 326001, India

[3] National Centre for Microbial Resource, National Centre for Cell Science, SP Pune University Campus, Pune 411007, India

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the fungus was obtained by hyphal tip transfer under aseptic conditions. At least ten Petri dishes of PDA were prepared by inoculating each with a 5 mm disc of actively growing mycelium of *Ganoderma* and kept at 28 ± 1 °C till further use (Mawar et al. 2020).

Eighteen soil samples were collected randomly from the rhizosphere of *Ganoderma* infected *P. cineraria* trees from different sites (Table 1). The samples were packed in zip locked bags and stored in a container with icepacks during transportation to the laboratory. From each sample, a serial dilution of the soil suspension from 10^{-1} to 10^{-6} was prepared. An aliquot of 100 µl of each dilution was spread evenly on the surface of actinomycete isolation agar (AIA) using an L- shaped glass spreader. All the plates were incubated at room temperature for 7–12 days. Colonies formed on the AIA plates were sub-cultured on starch casein agar media to obtain pure culture of the strain AZAC-1 (Waithaka et al. 2017).

Rhizospheric soil samples of ten *Ganoderma* infected Khejri trees from different sites were collected and used for bacterial isolation (Bajoria et al. 2008). Soil samples were air dried and 10 g of soil was mixed thoroughly with 100 ml of sterilized distilled water in 250 ml conical flask. The flasks with the soil suspension were shaken for 4 h. After heating at 80 °C for 15 min, a 1 ml aliquot of the soil suspension was poured into 9 ml of sterilized water in a 15 ml culture tube, vortexed thoroughly and serially diluted upto 10^{-7}. A100µl aliquot of $10^{-3} \times 10^{-5}$ and 10^{-7} dilutions were spread plated onto Petri dishes containing nutrient agar medium. After incubation for 24 h at 28 ± 1 °C, each Petri dish was inoculated with a 2 mm disc of actively growing *Ganoderma* mycelium. On the basis of clear zone of inhibition, bacterial colonies were selected and transferred onto nutrient agar media by the quadrant streaking method to isolate pure colonies. Scattered and isolated whitish flat pure colonies were picked gently and streaked onto nutrient agar dishes to obtain pure cultures of the most potential bacterial isolate AZ-11as a biocontrol agent for further processing.

Antagonistic activity of the isolated bacteria was confirmed by dual culture assay on PDA. Mycelium discs of *G. lucidum* (5 mm) was taken from the periphery of actively growing pure culture and placed in 90 mm PDA Petri dish. After 2 days, the promising antagonist actinomycete bacterial culture (10^{-7} cfu/ml) was streaked 30 mm away from the *G. lucidum* disc. The radial growth of the pathogen was measured after 6–7 days of incubation at 28 ± 2 °C and the percent inhibition of radial growth (PIRG) was calculated following Bivi et al. (2010). Plates with only *G. lucidum* served as controls. Each assay was repeated twice. The bacterium was streaked at the centre of the Petri dish and the test pathogen was placed at other side. Six Petri dishes served as six replications. These plates were incubated at 28 ± 2 °C and observations were recorded on mycelial growth of the pathogen at 24 h intervals upto120 h. The colony diameters of pathogen in control dishes were also recorded. The percent inhibition of growth of pathogen was calculated. The assay was repeated twice.

DNA was extracted from 48 h old culture of the isolates grown in nutrient broth. Cells were harvested by centrifuging the broth culture for 3 min at 13,000 rpm. After removal of supernatant the pellet was washed in 1 ml of re-suspension buffer (pH = 8), centrifuged again for 2 min, then placed on ice after removal of supernatant. The pellet was placed in a 1.5 ml centrifuge tube with 100 µl TE (Tris–Cl 10 ml M, Na-EDTA 1 ml M, pH = 8) and homogenized thoroughly by vortexing. The bacterial cells were lysed by sarcosyl and guanidinium thiocyanateon an ice bath. The solution was gently shaken by hand until it turned viscous. Further, 250 µl of ice-cold ammonium acetate (7.5 M) was added and the solution was shaken vigorously. Finally, 250 µl ice cold chloroform/isoamyl alcohol (24:1 ratio) was added and the solution was centrifuged for 10 min at 13,000 rpm till upper phase was clear. 700 µl of the upper clear layer was transferred into sterile Eppendorf tubes and 400 µl of cold isopropanol was added. DNA was precipitated by centrifugation at 13,000 rpm for 10 min. The pellet was washed thrice with 180 µl of ethyl alcohol (70%) and centrifuged at 13,000 rpm for 1 min. The DNA pellet was dissolved in 100 µl of 1X TE buffer (pH = 8) and used for amplification and sequencing.

For 16S rRNA gene amplification, DNA of the two isolates was used in PCR using the universal primers 27f

Table 1 Bacterial isolates of different rhizospheric soils collected from Jodhpur, Rajasthan

Isolates	Location	Host
AZBAC-1	Beejvadia, Jodhpur	*P. cineraria*
AZKAC-1	Khejarli, Jodhpur	*P. cineraria*
AZAC-1	CR Farm CAZRI	*P. cineraria*
AZ-1,AZ-2,AZ-3,AZ-4,AZ-5,AZ-6, AZ-7, AZ-8 AZ-9,AZ-10,AZ-11,AZ-12		
AZRKD-1	Rajor ki Dhani, Jodhpur	*P. cineraria*
ACNKT-1	Neem Ka Thana, Sikar	*P. cineraria*
AZLAC-1	Lordi-Dejnagra, Jodhpur	*P. cineraria*

(5′AGAGTTTGATCCTGGCTCAG3′) and 1492r (5′GGT TACCTT GTTACGACTT3′) (Lane 1991) with the following reaction conditions: initial denaturation at 97 °C for 4 min, followed by 35 cycles (94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min) and a final elongation step at 72°C for 5 min. Amplified products were separated on 0.8% (w/v) agarose gel and stained with ethidium bromide. The PCR product was further purified by PEG-NaCl precipitation and used for sequencing.

Sequencing was carried out at the National Centre for Microbial Resource (NCMR), Pune using additional internal primers so that each base was read at least twice. Sequences were then checked manually for error correction followed by contig assembly in SeqManPro v7 (DNAStar). Assembled sequences were used for identity match at EzBioCloud, a curated 16S database for microbial identification (Yoon et al. 2017). EzBioCloud uses a two-step approach wherein similar sequences are found first followed by the calculation of taxonomically meaningful pairwise sequence similarity values. This ensures that the top hit in the taxonomically validated database is the closest neighbour of the query sequence. For further validation of the identity, phylogenetic analysis of the sequences belonging to the closest hits for each strain was carried out in MEGA X (Kumar et al. 2018). The evolutionary history was inferred using Maximum Likelihood, Minimum Evolution, Maximum Parsimony and the Neighbor-Joining methods with 1000 replicates. The evolutionary distances were computed using the Kimura 2-parameter method in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution.

Bio-efficacy of cell free culture filtrates of BCAs was evaluated in vitro by standard poisoned food technique against *G. lucidum* in order to ascertain release of any metabolites by BCAs inhibiting *Ganoderma* in culture. Conical Erlenmeyer flasks (500 ml) containing 250 ml of sterilized nutrient medium was inoculated separately with 5 ml of 7 day-old culture of isolates AZ-11and AZAC-1. The flasks containing BCAs were incubated at 28 ± 2 °C for 10 days. After incubation, the broth culture was mixed thoroughly by hand blender and the conidial suspension was harvested using Whatman filter paper No. 1 and Sartorius Minisart Syringe filter was used for spore-free filtrate for inoculation. The cell free culture filtrates (3, 5, 7 and 10 ml) were added to the conical flasks containing 30 ml of Czapek's dox broth (CDB) and mixed well and then 5 mm disc of 7 days old culture of *G. lucidum* was inoculated in each flask. Four flasks of each antagonist were used as four replications for each concentration of filtrate. Flasks inoculated with only *G. lucidum* served as the control. All inoculated flasks were incubated at 28 ± 2 °C and after 7 days of growth, mycelial weight was recorded after drying the harvested mycelium at 60 °C for 48 h.

The isolate AZAC-1 was identified as *Streptomyces* sp. The assembled quality checked 16S rRNA gene sequence of AZAC-1 was 1377nt long (Gene Bank Accession number: MK459414).In EzBioCloud analysis, the 16S rRNA gene sequence of strain AZAC-1 showed 99.78% similarity to *Streptomyces mexicanus* strain CH-M-1035(T) (AF441168) with only 3 nucleotide differences as compared to 12 differences with the second closest neighbour *Streptomyces thermoviolaceus* subsp. *apingens* DSM 41,392(T) (Z68095) with 99.12% similarity. The near full-length sequence, a very high degree of similarity and very few differences with the closest neighbour in the database clearly suggested that the strain could be identified as *Streptomyces mexicanus.* This identity was also supported by the phylogenetic clustering of the strain AZAC-1 with its closest neighbour (Fig. 1a and Supplementary Figure S1). However, in the absence of MLSA data, the use of a single taxonomic marker and the lack of data obtained from other molecular identification techniques, we have conservatively designated the strain AZAC-1 as *Streptomyces* sp.

In contrast, the isolate AZ-11 was identified as *Bacillus* sp. and it required a much elaborate analysis. The assembled quality checked 16S rRNA gene sequences of AZ-11 was 1433 nt long (Gene Bank Accession number:MH304296). In EzBioCloud analysis, the 16S rRNA gene sequence of strain AZ-11 showed 99.93% similarity with a single nucleotide difference with three different species of *Bacillus*: *Bacillus tequilensis* KCTC 13,622(T) (AYTO01000043), *Bacillus subtilis* subsp. *inaquosorum* KCTC 13,429(T) (AMXN01000021) and *Bacillus cabrialesii* TE3(T) (MK462260). Further phylogenetic analyses with the top 25 sequences using multiple methods showed the clustering of strain AZ-11 with *Bacillus tequilensis* KCTC 13,622(T) with high bootstrap supports in both Neighbor-Joining and Minimum Evolution based trees (Fig. 1b and S2). In contrast, the strain also clustered with *Bacillus subtilis* sub sp.*subtilis* (Fig. S2a), *Bacillus subtilis* sub sp.*inaquosorum* (Fig. S2b) and *Bacillus mojavensis* (Fig. S2c). Thus, the identity remained inconclusive. We therefore, reconstructed the phylogeny using multiple reference sequences of these three species. We ensured that the sequences used were either derived from the whole genome or from near-full length PCR amplicons from type or other standard strains only. Given these parameters, very few sequences qualified these criteria and could be included (2 each for *Bacillus tequilensis* and *Bacillus subtilis* sub sp. *inaquosorum*, 3 for *Bacillus subtilis* subsp. *subtilis* and 4 for *Bacillus mojavensis*). The strain AZ-11 clustered with *Bacillus tequilensis* as well as *Bacillus subtilis* subsp. *subtilis* originating from the same node (see Fig. 1b). Given the continued in consistencies in clustering, we conservatively designate the strain as *Bacillus* sp. A detailed investigation to accurately ascertain the taxonomic identity of this strain

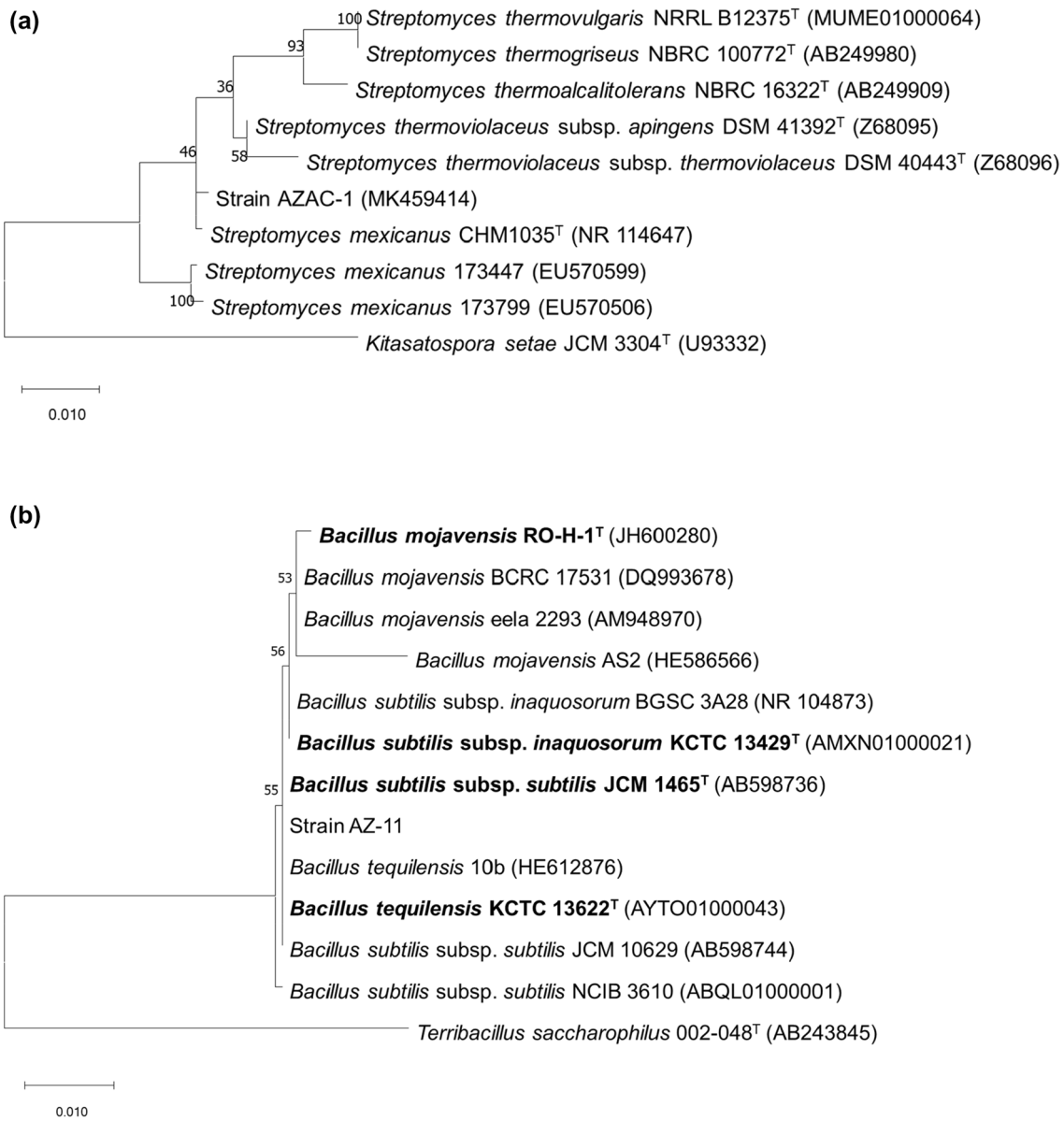

Fig.1 **a** Maximum-Likelihood based phylogenetic tree for the strain AZAC-1 with multiple strains of *S. mexicanus,* the closest neighbour in EzBioCloud. The tree with the highest log likelihood (− 2586.69) is shown. There were a total of 1333 positions in the final dataset. *Kitasatosporasetae* JCM 3304 ^{T} (U93332) was used as an outgroup. **b** Maximum-Likelihood based phylogenetic tree for the strain AZ-11 with multiple strains of the three closest neighbours in EzBioCloud. The type strains are highlighted in Bold. The tree with the highest log likelihood (− 2237.68) is shown. There were a total of 1224 positions in the final dataset. *Terri bacillus saccharophilus* 002-048 ^{T} (AB243845) was used as an outgroup

to species level is being pursued and is beyond the scope of this study.

Both *Streptomyces* and *Bacillus* are large bacterial genera with 664 and 203 validly published species, respectively (record as per List of Prokaryotic Names with Standing in Nomenclature, https://lpsn.dsmz.de as of 24th Aug 2020). Further detailed phylogenetic analysis of the 16S rRNA gene sequences of the top 25 closest type sequences observed inEzBioCloud for each strain was carried out using multiple methods and the identity of the strains was confirmed based upon a high bootstrap support (Fig. 1). For strain AZAC-1, the top25 sequences showed 99.78—98.16% similarity accounted by 3 to 25 differences, whereas for strain AZ-11 and the sequence similarity ranged from 99.93 to 96.84%

accounted by 1 to 45 differences spanning the length of the query sequence. Regardless, the clustering of the strains with their respective closest neighbours was confirmed by more than one method. Given the extent of similarity, the degree of differences with the closest neighbour in the database and the phylogenetic clustering of the strains, the taxonomic identity of the strains have been accurately determined to the extent reported.

In dual culture test between *Streptomyces* sp. strain AZAC-1 and the test pathogen, an inhibition zone of 1.5 – 2.0 mm was recorded in six Petri dishes where four Petri dishes had more than 1.5 mm inhibition zone (Fig. 2). Dual culture characteristic was studied for the antagonist actinomycete bacterial culture which showed highest PIRG against *G. lucidum*. In a similar study, Lim et al. (2018) isolated *Streptomyces* sp.A19, which was found highly antagonistic to *G. boninense* causing basal stem rot in oil palm. Several antimicrobial compounds isolated from their interaction were attributed for inhibiting the pathogen under laboratory conditions. Similarly, Shariffah-Muzaimah et al. (2015) isolated several isolates of *Streptomyces* that were antagonistic to *G. boninense* from Malaysia. Soil beneath the canopy of *P. cineraria* trees has higher microbial population compared to soil away from tree due to high organic matter (Purohit et al. 2002). Actinomycetes have been demonstrated to grow extensively in soils rich in organic matter and moisture content (Jayasingh and Parkinson 2007). Actinomycetes are a group of microorganisms with a huge potential as bio control agents due to their ability to produce promising secondary metabolites with biological activities such as antibiotics, antifungal, plant growth factor and enzyme inhibitors (Berdy 2005).

The bacterial strain, *Bacillus* sp.AZ-11was also found as effective as AZAC-1 where an inhibition zone of more than 5 mm between the strain AZ-11and test pathogen was recorded with 35.6% reduction in *Ganoderma* growth compared to control Petri dishes having *G. lucidum* only (Fig. 2). Several species of the genus *Bacillus* have been reported as potential BCAs. For instance, the bacterium *B. tequilensis* is reported to manage root knot nematode when co-inoculated with *Trichoderma harzianum* (Tiwari et al. 2017).Thus, this bacterium can have dual advantage in irrigated pockets where several vegetables suffer from root knot nematode. Incidentally, *B. tequilensis* is also known to solubilise zinc for increased yield of wheat and soybean (Khande et al. 2017). In arid region, one strain of *B. firmus* has also been reported as specific antagonist to *Macrophomina phaseolina* (Lodha et al. 2013) in earlier studies. Bajoria et al. (2008) isolated *B. subtilis, B. cereus, B. pumilus* and *B. sphaericus* from hot arid region having biocontrol potential against plant pathogenic fungi. Therefore, the isolated antagonistic bacterial strains will be potentially very useful and yield effective results in further field studies for management of this terrific pathogen (Fig. 3).

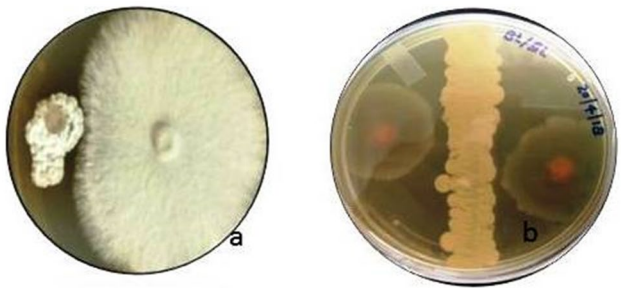

Fig. 2 Inhibition zone of *S. mexicanus* (**a**) and *Bacillus* sp. (**b**) against *G. lucidum*

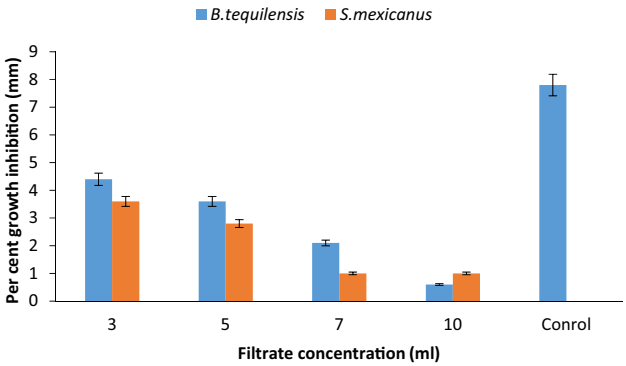

Fig.3 Mycelial growth of *Ganoderma* in presence of cell- free filtrate (3–10 ml) of *S. mexicanus* strain AZAC-1and *Bacillus tequilensis* strain AZ-11

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42360-021-00327-1.

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