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Isolation and Molecular Characterization of Bacterial Strains with Antifungal Activity from Termite Mound Soil

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A B S T R A C T

We looked for bacterial strains with antifungal activity in the termite mound soil. We have detected good antifungal activity of bacterial strains which were selected on the basis of prescreening procedure to search for plant growth promoting traits. Twenty one bacterial isolates were tested against the pathogens *Sclerotium rolfsii, Alternaria sp., Colletotrichum truncatum, Rhizoctonia solani* and *Fusarium oxysporum*. Only 10 out of 21 tested bacterial isolates showed antifungal activity against *Fusarium oxysporum*, 8 showed against *Alternaria brassicae* and *Rhizoctonia solani*, only one isolate showed against *Sclerotium rolfsii*. While, all the 21 tested bacterial isolates showed activity against *Colletotrichum truncatum*. Four efficient isolates named as NTS 65, BTS 14, BTS 16 and NTS 20 were further screened on the basis of 16S rRNA typing and these were identified as *Staphylococcus saprophyticus, Bacillus methylotrophicus* and *Bacillus sp.* The sequences of these isolates were submitted with NCBI gene bank.

Introduction

Termites process high quantities of material in their mound building activity, thus influencing the soil properties as compared to the surrounding soil (Wood, 1998). Soil of termite mounds exhibits a higher proportion of fine particles, which they transport from the deeper to upper soil horizons. In some countries, termite mound soil has been used to enrich the crop field with available nitrogen, total phosphorous and an organic carbon than the adjacent soil (Breuning *et al.,* 2005).

Although, usually considered as pests, termites can be valuable not only in forest ecosystem but also in organic farming (Arnold *et al.,* 2017). They are great decomposers of wood and plant debris, they aerate the soil and add nutrients to it. Plants also take up nutrients very easily from termite mound soil. Termite soil is providing a viable option to local farmers who can't afford to buy expensive inorganic fertilizers.

It has been reported that bacterial and microbial populations in termitaria soil are higher than the adjacent soil (Spain 2004) and some of them have been associated with the suppression of fungal diseases. Termite mound have been used in organic farming to control some diseases, and also as a source

with potential to enhance crop production and manage plant pathogens (Rupela, 2003).

Plant pathogens are major and chronic threat to food production and ecosystem stability worldwide. Over the past few decades, producers became more and more dependent on synthetic agro-chemicals for crop protection.

However, increased and indiscriminate use of synthetic agro-chemicals cased several negative impacts on environment and human health. Use of microbial inoculants is one of the most acceptable and eco-friendly approach to solve the problem of plant pathogens. Antagonistic activity of bacterial strains has been recognized as major factor in the suppression of many phytopathogens (David *et al.*, 2015).

Thus the present study entitled was undertaken to demonstrate the role of termite mound soil.

In this study we searched for bacterial species with antifungal activity in the termite mound soil. We have detected good antifungal activity of bacterial strains which were selected on the basis of prescreening procedure to search for plant growth promoting traits.

Twenty one bacterial isolates were tested against the pathogens *Sclerotium rolfsii*, *Alternaria sp*., *Colletotrichum truncatum*, *Rhizoctonia solani*, and *Fusarium oxysporum*. Four efficient isolates named as NTS 65, BTS 14, BTS 16 and NTS 20 were further screened on the basis of 16S rRNA typing and these were identified. The 16S ribosomal gene sequence from the isolates NTS 65, BTS 14, BTS 16 and NTS 20 were amplified with polymerase chain reaction (PCR) and sequenced, showing identity with the as *Staphylococcus saprophyticus, Bacillus methylotrophicus* and *Bacillus sp.*

Materials and Methods

Experiment site

The present study was conducted in the Department of Microbiology, CSK HPKV, Palampur.

Sample collection and bacterial isolation

Samples for present study were collected from different regions of Himachal Pradesh as well as from Model Organic Farm, Department of Organic Agriculture, CSK HPKV, Palampur. Termite mound soil samples were collected in sterilized polythene bags from different district of Himachal Pradesh. These samples were brought to the laboratory and processed immediately or kept at refrigerator conditions for further analysis. Soil sample weighing 1 g was taken and transferred to 9 mL sterilized dilution blank under aseptic conditions and then agitated for 15 minutes on a shaker which provided 10^{-1} dilution and further serial dilutions were made from this accordingly. Microbial load was determined by using standard plate count technique (Wollun, 1982) by employing different media like of nutrient agar, Jensen's Agar and Pikoskaya's agar etc.

Evaluation of antagonism against plant fungi

In order to test the antifungal activity of isolates, a loop full of 48 hours old culture of each isolate was streaked at 4 sides, a little below at the centre of PDA plates, and incubated for overnight at 28 ± 2°C, to check for contamination. Mycelia disk of 4 days old culture of each test fungal pathogen was placed simultaneously in between the streaks (2mm apart from fungal disk). A control inoculated with test pathogen only was kept for comparison. The plates were incubated at 28 ± 2°C and percent growth inhibition was calculated according to Vincent (1947).

Percent inhibition = C – T/ C

Where,

C = radius of fungus in control (mm)
T = radius of fungus in treatment (mm)

Characterization and identification of selected bacterial isolates

Bacterial isolates were identified on the basis of morphological (type, shape, staining, colony characteristics) and biochemical characteristics (Oxidase, Catalase, Utilization of Sugar, Indole, MR-VP, Citrate, Urease, etc.) according to the standard method descrived in Bergey's Manual of Systematic Bacteriology (Krieg and Holf, 1984) and laboratory manual of Basic Microbiology (Kanwar *et al.*, 1997)

Molecular characterization of efficient bacterial isolates

Molecular characterization of efficient isolates was done by 16S rRNA gene sequencing method, and these sequences were blasted by using online NCBI Blast Program. *http://www.ncbi.nih.gov/blast*. Dendrogram was prepared by using Mega 4.1 software.

Maintenance of isolates

Soft agar

All the isolates were stocked in soft agar (0.5% agar) as well as on plates and slants of nutrient agar and potato dextrose agar. After the growth of isolates in incubator at 28 ± 2°C, plates and tubes were preserved at 4°C under refrigerated conditions.

Glycerol stocks

Glycerol stock cultures of all the isolated microorganisms were prepared and stored at -20°C. Cultures were grown in 50 mL nutrient broth for 12 hours and 0.8 mL aliquots were added to 0.2 mL sterile 75% Glycerol in 2 mL vials.

Results and Discussion

Antifungal activities of isolated bacteria species

Antifungal activity of all bacterial isolates from termite mound soil was checked by Agar streaked method. Among these isolates, 21 isolates showed antagonistic activity against phytopathogen *Colletotrichum truncatum*, 10 against *Fusarium oxysporum*, 8 against *Rhizoctonia solani* and *Alternaria brassicae* and one against *Sclerotium rolfsii* (Table 1, Fig. 2 and 3).

In case of antifungal activity against *Colletotrichum truncatum*, the maximum percent inhibition was showed by BTS 16 (59.52%) followed by NTS 65 (58.73%), BTS 14 (55.55%) and NTS 20 (50.00%). The maximum antifungal activity against *Fusarium oxysporum* was given by BTS 14 (67.44%) followed by NTS 65 (66.67%), BTS 16 (65.89%), PTS 22 (62.79%) and PTS 39 (61.24%). In case of *Rhizoctonia solani*, the maximum inhibition was given by BTS 16 (63.49%), BTS 14 and PTS 39 (60.31%) followed by NTS 65 (58.73%).

Whereas, in case of *Alternaria brassicae* the maximum antifungal activity in terms of percent inhibition was given by NTS 20 (65%), NTS 65 (63.33%), BTS 14 (61.66%) and PTS 4 (60.83%).

NTS 20, NTS 65, BTS 14 and BTS 16 have found to be efficient isolates in terms of antifungal activity against selected phytopathogens and thus these can further be exploited as biocontrol agents in organic farming (Ramesh *et al.*, 2010).

Table.1 Antifungal activity of bacterial isolates against phytopathogens

Isolates	Radius (mm)	Radius (control)	% inhibition
	Colletotrichum truncatum		
NTS 65	17.30	42	58.73
NTS 20	21.00	42	50.00
BTS 14	18.67	42	55.55
BTS 16	17.00	42	59.52
Fusarium oxysporum			
NTS 65	14.33	43	66.67
NTS 20	18.00	43	58.14
PTS 22	16.00	43	62.79
BTS 14	14.00	43	67.44
BTS 16	14.67	43	65.89
PTS 39	16.67	43	61.24
Rhizoctonia solani			
BTS 16	15.33	42	63.49
BTS 14	16.67	42	60.31
PTS 39	16.67	42	60.31
NTS 20	19.33	42	53.97
NTS 65	17.33	42	58.73
Alternaria brassicae			
BTS 16	19.00	40	52.50
BTS 14	15.33	40	61.66
NTS 65	14.67	40	63.33
NTS 20	14.00	40	65.00
Sclerotium rolfsii			
PTS 22	17.00	43	60.47

Fig.1 Phylogenetic tree of molecularly characterized isolates

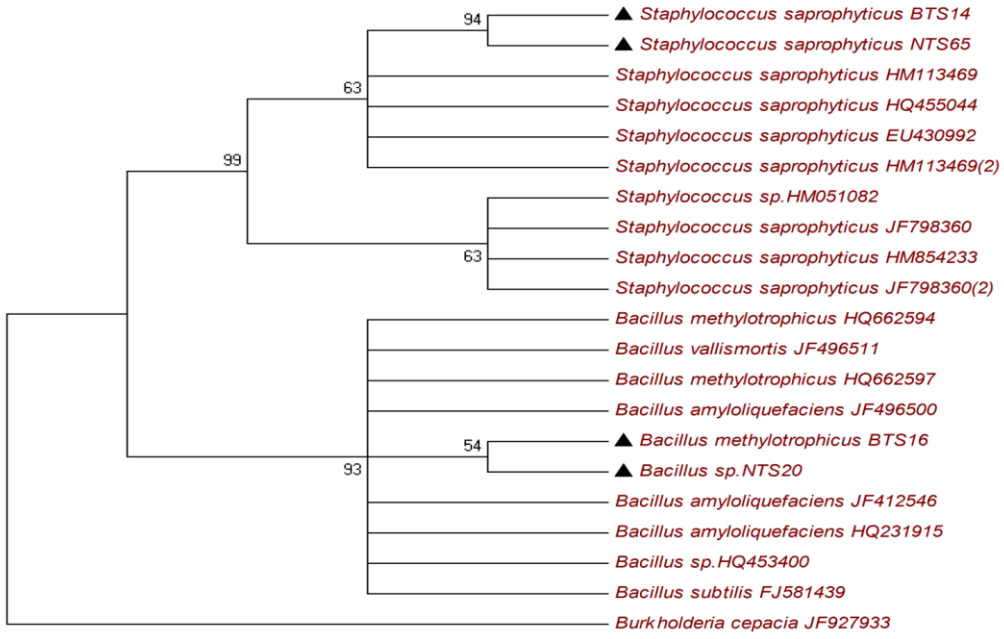


Fig.2 Antifungal activity of bacterial isolates against *Colletotrichum truncatum* (a) and *Fusarium oxysporum* (b) bacterial isolates showing inhibition against *Colletotrichum truncatum* (c-d) and *Fusarium oxysporum* (e-f)

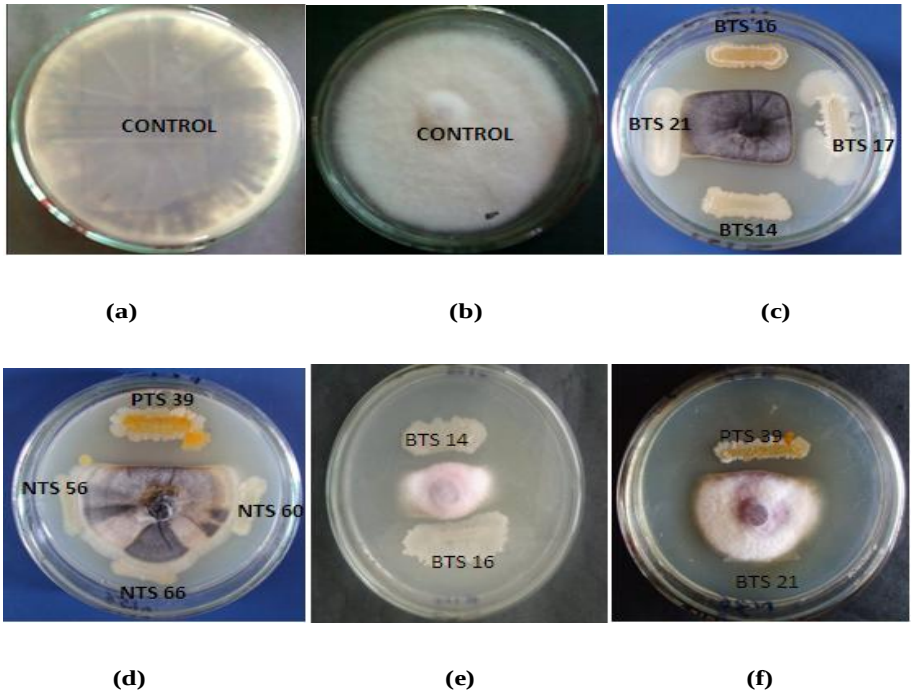
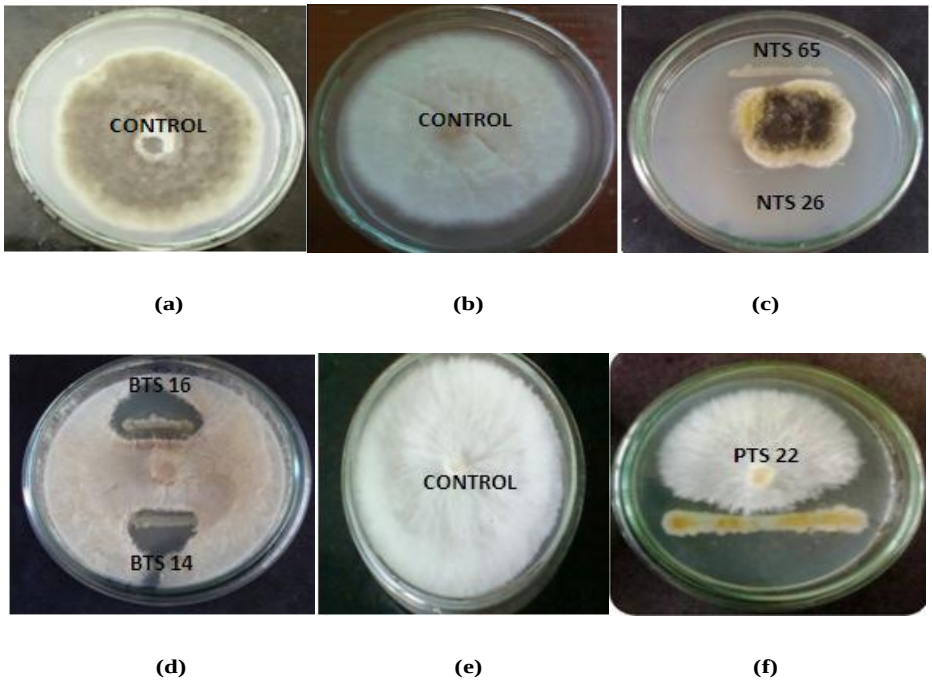


Fig.3 Antifungal activity of bacterial isolates against *Alternaria brassicae* (a*) Rhizoctonia solani* (b) *Sclerotium rolfsii* (e) bacterial isolates showing inhibition against *Alternaria sp*. (c) *Rhizoctonia solani* (d) and *Sclerotium rolfsii* (f)



Exhibition of antifungal activity could be attributed to production of substances like siderophore, HCN, organic acid etc. and these observations are in correspondence with the study of Srivastava *et al.,* (2004) and Bano *et al.,* (2004).

Molecular characterization of efficient bacterial isolates

Those bacterial isolates which showed maximum biological activities were further characterized on the basis of molecular characteristics. After 16s rRNA gene sequencing, the sequences obtained were submitted to NCBI GeneBank Nucleotide Database.

Analysis of the sequences, using BLASTN algorithm of NCBI, identified the isolates were found to be belonging to two bacterial species. On the basis of high sequence similarity shown to a particular bacterial species already available in the GeneBank. Isolate NTS 65 and BTS 14 have shown sequence similarity with *Staphylococcus saprophyticus strain* (JF 798360, HQ 455044, EU 430992 and HM 113469). Whereas, BTS 16 have shown sequence similarity to *Bacillus methylotrophicus* (HQ 662597, HQ 662594). And NTS 20 have shown sequence similarity with *Bacillus* species (HQ 453400) as shown in dendrogram. NCBI gene Bank allotted Accession number to two molecularly characterized cultures, NTS 65 (JN 162677) and BTS 14 (JN 162676).

The phylogenetic tree prepared with 16S rRNA gene sequence of *Burkholderia capacia* as an outgroup bacterium and 16 other gene sequences of bacteria selected from NCBI, these 4 isolates under study were found to belonging to three groups. Group I was identified as *B. methylotrophicus*, group II as *Bacillus species* and group III identified as *staphylococcus saprophyticus.*

Many workers have identified bacterial strains belonging to genus *Bacillus* and *Staphylococcus* associated with the soils by using specific primer sets and 16S rRNA gene analysis coupled with conventional identification techniques (Pamela *et al.,* 2010).

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