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# Diversity and Antimicrobial Potential of Cultivable Endophytic Actinobacteria Associated With the Medicinal Plant *Thymus roseus*

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# Diversity and Antimicrobial Potential of Cultivable Endophytic Actinobacteria Associated With the Medicinal Plant *Thymus roseus*

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# Introduction

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- Introducing and importance of endophytic actinobacteria
  - Microbial biodiversity
  - Plant natural defenses
  - Potential in pharmaceutical, agricultural, and food industries applications.
- The reason for choosing the *Thymus roseus*
  - Traditional Chinese medicinal herbs
  - Strong adaptive capability for a variety of particular habitats
  - Essential oils with pharmaceutical application

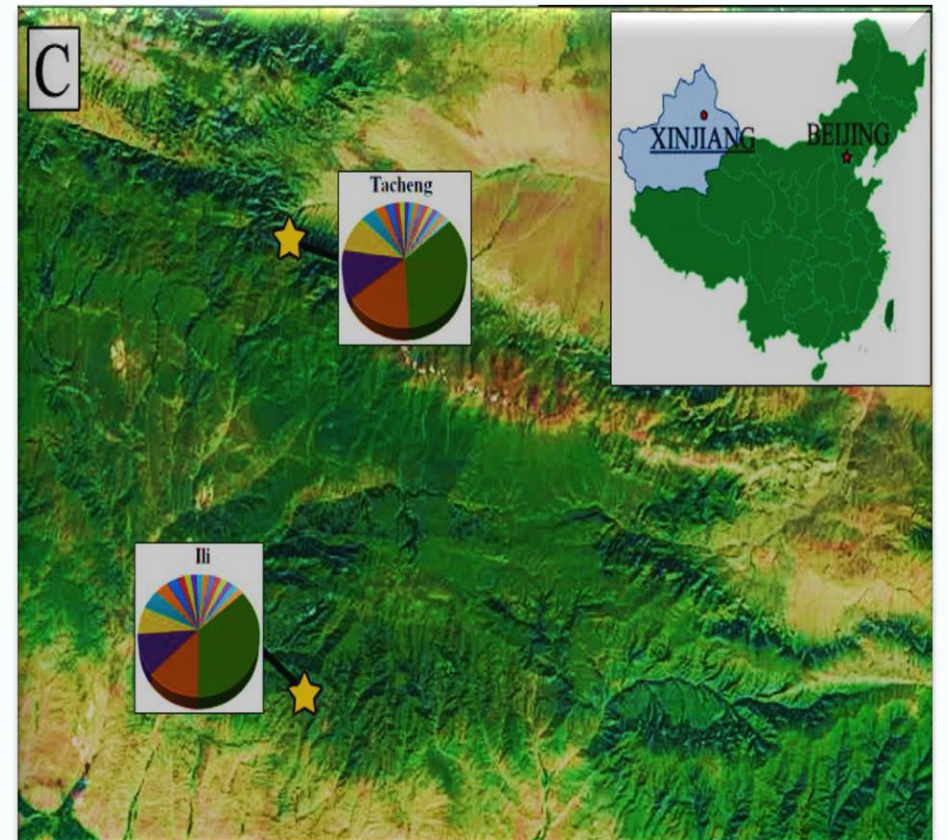
## The aim of study

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1. To isolate and identify endophytic actinobacteria by a culture dependent method.
2. Analyzing the species richness and distribution pattern among the different plant tissues and locations.
3. Comparing the effectiveness of plant tissue pretreatment on the isolation of endophytic actinobacteria.
4. Determining their antimicrobial capability and to screen the isolates with the antibiotic biosynthetic genes *in vitro* conditions.

# Material And Methods

- Site Selection and Plant Sampling
  - Ili and Tacheng, China



## Surface Sterilization

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- First; each plant sample was washed with running tap water and separated into stem, root, and leaf segments. Then washed by Sonification for 30 min at 45 kHz to remove the dislodge soil and organic matter. Then;
  1. Plant segments were rinsed in 0.1% Tween 20 for 1 min
  2. Immersing in 5% sodium hypochlorite (NaOCl) for 4-6 min
  3. Washed for 10 min in 2.5%  $\text{Na}_2\text{S}_2\text{O}_3$
  4. Soaked in 70% ethanol for 4-6 min
  5. Rinsing in 10%  $\text{NaHCO}_3$  for 10 min

## Pretreatment for isolation

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- Three kinds of pretreatment were performed:
  1. Surface sterilized plant tissues were directly taken to isolation.
  2. Freezing treatment involved plant tissues being frozen at  $-80^{\circ}\text{C}$  for 2 weeks.
  3. High temperature treatment involved plant tissues drying at  $100^{\circ}\text{C}$  for 20 min.

## Isolation of Endophytic Actinobacteria

- For each pretreated sample, about 1 g of tissue homogenate was weighed and macerated along with 9 mL sterile double distilled water. After precipitation for 10 min at room temperature, the supernatant was serially diluted ( $10^{-2}$  and  $10^{-3}$ ), and a suspension of 0.2 mL was spread onto ten different selective isolation media.
- Nystatin,  $K_2Cr_2O_7$  and nalidixic acid were used for inhibit the growth of bacteria and fungi.
- All agar plates incubated at  $28^{\circ}C$  for 45 days.



## Isolation media

**TABLE 1** | Composition of the media used for the isolation of endophytic actinomycetes from medicinal plants.

Medium	Composition	References
M1	Tap water yeast extract (TWYE): yeast 0.25 g; K <sub>2</sub> HPO <sub>4</sub> 0.5 g; Agar 15 g; pH 7.2; distilled water 1000 mL	El-Shatoury et al. (2006)
M2	Cellulose-proline: cellulose 2.5 g; sodium pyruvate 2.0 g; proline 1.0 g; KNO <sub>3</sub> 0.25 g; MgSO <sub>4</sub> ·7H <sub>2</sub> O 0.2 g; K <sub>2</sub> HPO <sub>4</sub> 0.2 g; CaCl <sub>2</sub> 0.5 g; FeSO <sub>4</sub> ·7H <sub>2</sub> O 0.01 g and agar 15 g; distilled water 1000 mL; pH (7.2–7.4)	Modified from Wang (2015)
M3	Sodium succinate-asparagine: sodium succinate 1.0; L-asparagine 1.0 g; KH <sub>2</sub> PO <sub>4</sub> 0.9 g; K <sub>2</sub> HPO <sub>4</sub> 0.6 g; MgSO <sub>4</sub> ·7H <sub>2</sub> O 0.1 g; CaCl <sub>2</sub> 0.2 g; KCl 0.3 g; FeSO <sub>4</sub> ·7H <sub>2</sub> O 0.001 g; agar 15 g; distilled water 1000 mL; pH 7.2	Modified from Wang (2015)
M4	Xylan-asparagine: xylan 2.5 g; asparagine 1 g; K <sub>2</sub> HPO <sub>4</sub> 0.5 g; KNO <sub>3</sub> 0.25 g; MgSO <sub>4</sub> ·7H <sub>2</sub> O 0.2 g; CaCl <sub>2</sub> 0.5 g; FeSO <sub>4</sub> ·7H <sub>2</sub> O 0.01 g and agar 15 g in 1000 mL distilled water; pH (7.2–7.4)	Qin et al. (2009)
M5	Sodium propionate-asparagine: sodium propionate 2 g; L-asparagine 1.0 g; NH <sub>4</sub> NO <sub>3</sub> 0.1 g; KCl 0.1 g; MgSO <sub>4</sub> ·7H <sub>2</sub> O 0.05 g; FeSO <sub>4</sub> ·7H <sub>2</sub> O 0.05 g; plant extract (100 g plants was boiled in 1 L distilled water for 1 h and then filtered and concentrated to 100 mL) 1 mL; agar 15 g; distilled water 1000 mL; pH 7.2	Qin et al. (2009)
M6	Histidine-raffinose: histidine 0.5 g; raffinose 2.5 g; K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O 1 g; MgSO <sub>4</sub> ·7H <sub>2</sub> O 0.5 g; FeSO <sub>4</sub> ·7H <sub>2</sub> O 0.01 g; CaCl <sub>2</sub> 0.02 g; distilled water 1000 mL	Vickers et al. (1984)
M7	Humic-vitamin (HV): humic acid 1 g; Na <sub>2</sub> HPO <sub>4</sub> 0.5 g; KCl 1.7 g; MgSO <sub>4</sub> ·7H <sub>2</sub> O 0.05 g; CaCl <sub>2</sub> 1 g; vitamins mixture 1 g; distilled water 1000 mL	Hayakawa (1990)
M8	Oatmeal agar (ISP3): oatmeal 20 g; saline standard solution 1 mL; distilled water 1000 mL	Shirling and Gottlieb (1966)
M9	Modified Gause No. I: starch 20 g; KNO <sub>3</sub> 1 g; K <sub>2</sub> HPO <sub>4</sub> 0.5 g; MgSO <sub>4</sub> ·7H <sub>2</sub> O 0.5 g; NaCl 0.5 g; FeSO <sub>4</sub> ·7H <sub>2</sub> O 0.01 g; distilled water 1000 mL	Shirling and Gottlieb (1966)
M10	Citrate agar: citric acid 0.12 g; NaNO <sub>3</sub> 1.5 g; K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O 0.4 g; Mg SO <sub>4</sub> ·7H <sub>2</sub> O 0.1 g; CaCl <sub>2</sub> ·H <sub>2</sub> O 0.05 g; EDTA 0.02 g; Na <sub>2</sub> CO <sub>3</sub> 0.2 g; agar 15 g; pH 7.2; distilled water 1000 mL	Zhang et al. (2013)

## Genomic DNA extraction

- Fresh bacterial biomass (50 mg) was resuspended in 35  $\mu\text{l}$  lysis solution (50 mM sucrose, 50 mM Tris-HCl pH 8.0, and 20 mM EDTA-Na) and 15  $\mu\text{l}$  20% SDS and then heated in a microwave oven for 90 s. The mixture was treated with 450  $\mu\text{l}$  DNA extraction solution (100  $\text{mmol.L}^{-1}$  Tris, 100  $\text{mmol.L}^{-1}$  EDTA, 200  $\text{mmol.L}^{-1}$  NaCl, 2% PVP, 3% CTAB, and pH 9.0) and then extracted twice with phenol/chloroform/isoamyl alcohol, followed by precipitation with 800  $\mu\text{l}$  ethanol and 80  $\mu\text{l}$  sodium acetate (3  $\text{mol L}^{-1}$  and pH 4.8–5.2). The DNA pellet was washed with 70% ethanol, air-dried, resuspended in 50  $\mu\text{l}$  deionized distilled water, and then stored.

## Taxonomic Characterization

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- 27F and 1492R were primers for 16S rRNA
- The 16S rRNA gene sequences were compared with Ezbiocloud1 and GenBank databases using BLAST software.
- Multiple sequence alignment was performed using the CLUSTAL X 1.83 program.
- Phylogenetic tree was generated by MEGA version 7.0 software.

## Antagonistic Assays of Antifungal Activities In vitro

- The tested fungi were *Fusarium oxysporum*, *Fulvia fulva*, *Alternaria solani*, *Fusarium oxysporum*, *Valsa malicola*, and *Valsa mali* by the plate confrontation method:
  - A fungal disk 5<sup>mm</sup> in diameter containing 7-day-old mycelial growth was placed at the center of a 9<sup>cm</sup> PDA plates. The four actinobacteria disks cultured on ISP2 medium for 4–7 days at 28<sup>C</sup> were placed onto the agar surface at four equidistant points, 2.5<sup>cm</sup> from the plate periphery. All agar plates were wrapped with parafilm and incubated at 28<sup>C</sup> for 3–5 days.
  - $I = (R_0 - R_i) / R_0 \times 100\%$

# Antagonistic Activity Against Human Pathogenic Bacteria

- The tested bacteria were *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella enteritidis*
  - The bacteria were cultured for 1 night in LB medium at 37°C. 5 mL of each culture was centrifuged at 5000 rpm for 10<sup>min</sup>. The pellets were resuspended in sterile DDH<sub>2</sub>O and density adjusted to 10<sup>8</sup> colony forming units CFU/mL. A total of 100 μL of the typical bacteria cell were inoculated and evenly spread by sterile cotton swaps onto the surface of the LB medium, and then four 5<sup>mm</sup> diameter pieces of sterile filter paper were placed on each corner of the agar plate. After this, 10 mL of each actinobacteria strain was added dropwise to the filter paper.

# Screening for Natural Product Biosynthetic Gene Clusters by PCR Method

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- Fifty-four strains showed antimicrobial activity against one or more indicator organisms and were used for screening for natural product biosynthetic gene clusters by PCR.
  - Three sets of degenerate primers targeting biosynthetic genes were used for PCR amplification: KSF and KSR, which target PKS-I; KSaF and KSaR, which target PKS-II KSa genes; and A3F and A7R, which target non-ribosomal peptide synthetase (NRPS) genes.

# RESULTS

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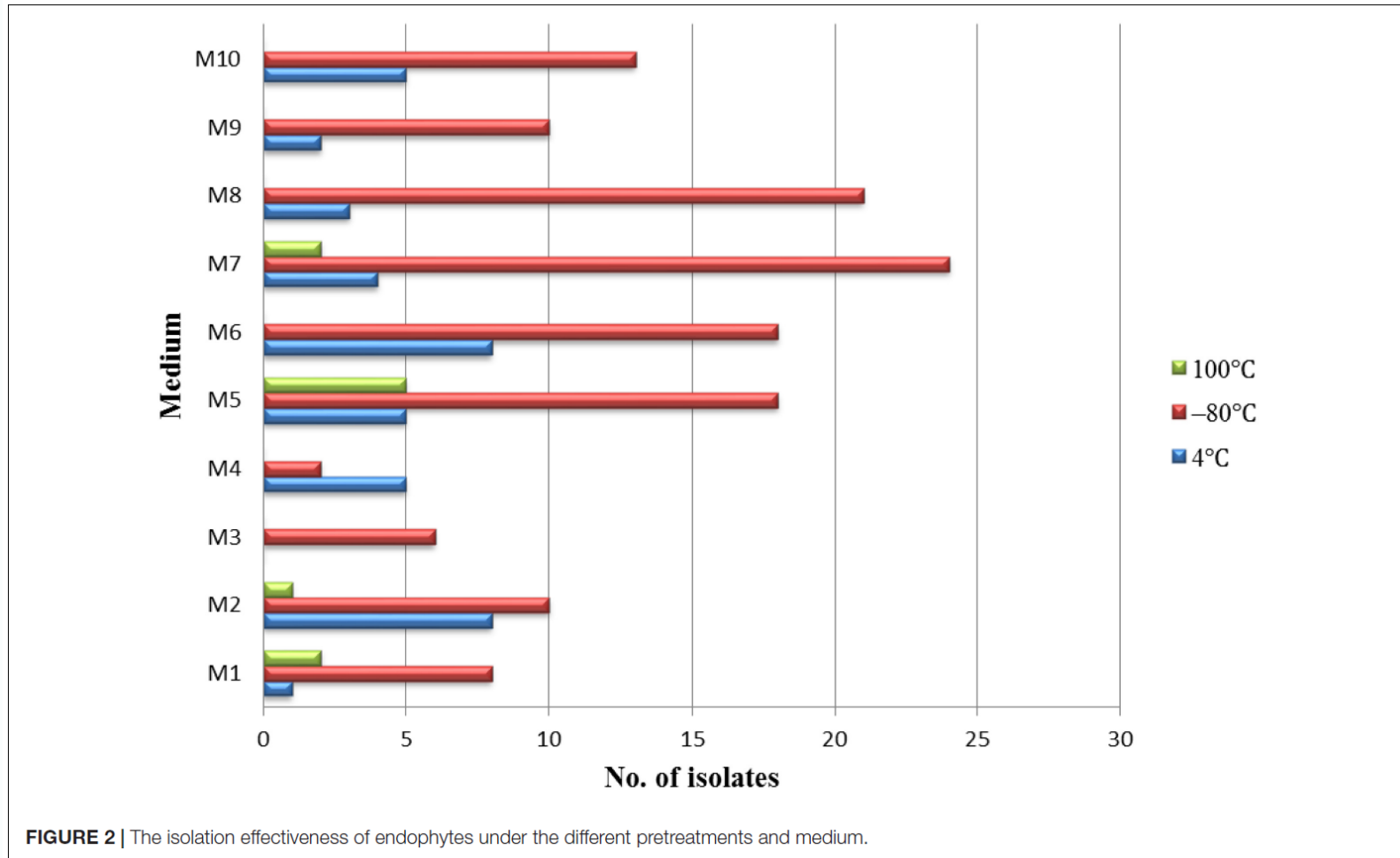
- Effectiveness of Surface Sterilization
- Pretreatment, Isolation Medium, and Effectiveness of Isolation
- Diversity and Tissues Specificity of Endophytic Actinobacteria
- Antimicrobial Activity of the Isolates
- Screening of Chitinase- and Siderophore-Producing Strains
- Screening of Isolates With Antibiotics Biosynthetic Genes

## Pretreatment, Isolation Medium, and Effectiveness of Isolation

A total of 181 endophytic actinomycetes were isolated:

128 (-80°C), 41 (4°C), 10 (100°C)

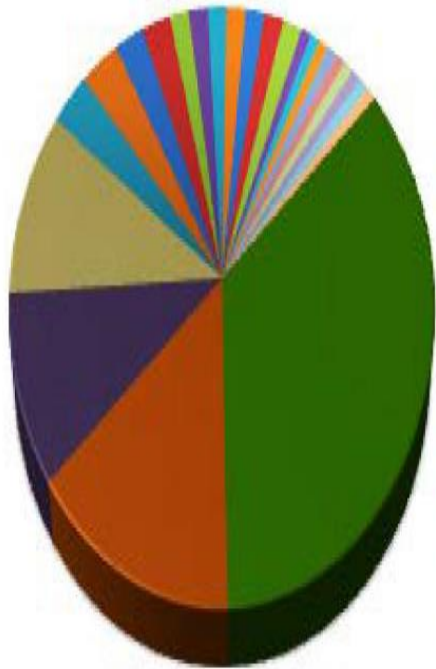
The majority of isolations were from HV (M7), sodium propionate agar (M5), raffinose-histidine agar (M6), and oatmeal agar medium (M8).



**FIGURE 2** | The isolation effectiveness of endophytes under the different pretreatments and medium.



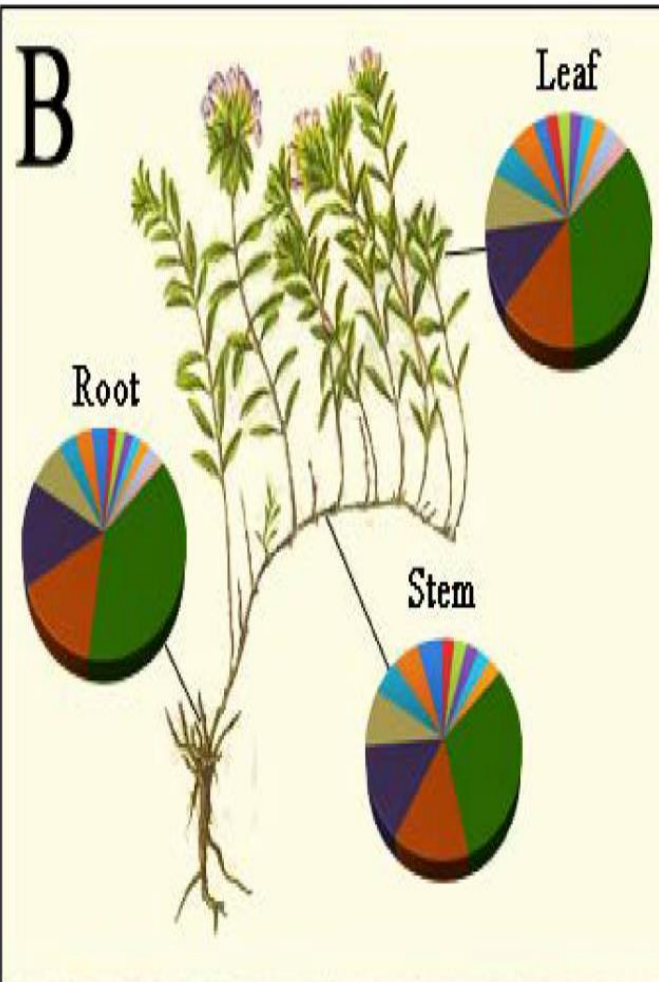
# A All sites



## Genus

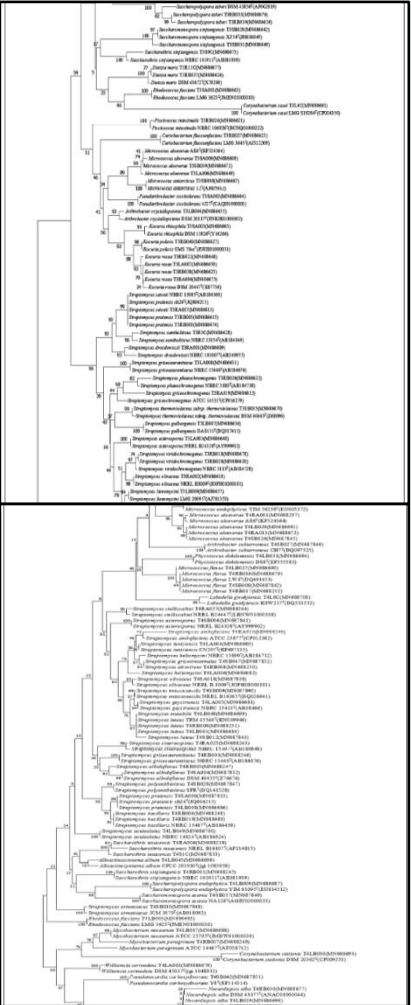
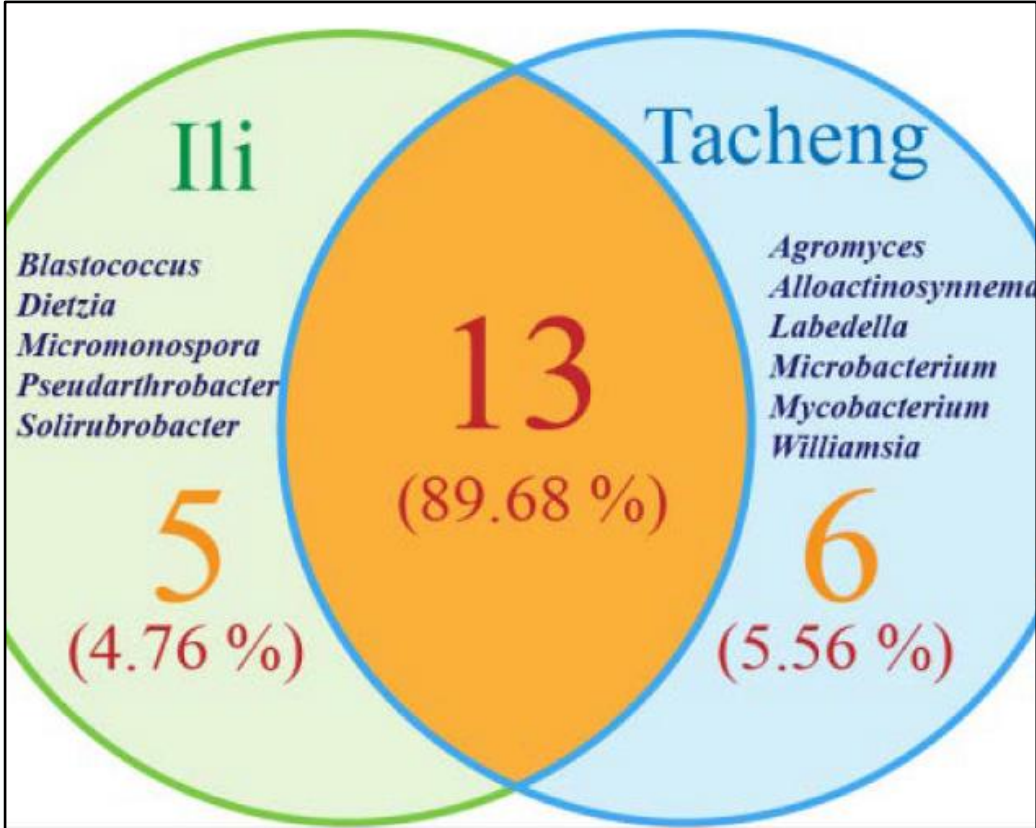
- |                          |                          |
|--------------------------|--------------------------|
| <i>Streptomyces</i>      | <i>Nocardopsis</i>       |
| <i>Micrococcus</i>       | <i>Kocuria</i>           |
| <i>Saccharothrix</i>     | <i>Saccharopolyspora</i> |
| <i>Saccharomonospora</i> | <i>Arthrobacter</i>      |
| <i>Rhodococcus</i>       | <i>Pseudonocardia</i>    |
| <i>Piscicoccus</i>       | <i>Mycobacterium</i>     |
| <i>Dietzia</i>           | <i>Curtobacterium</i>    |
| <i>Corynebacterium</i>   | <i>Solirubrobacter</i>   |
| <i>Pseudarthrobacter</i> | <i>Micromonospora</i>    |
| <i>Microbacterium</i>    | <i>Labedella</i>         |
| <i>Alloactinosynnema</i> | <i>Agromyces</i>         |

# B



Diversity and Tissues Specificity of Endophytic Actinobacteria

# Diversity



Based on 16S rRNA gene sequences 126 endophytic actinobacteria isolates were obtained that 55 and 71 isolates were from Ili and Tacheng sites, respectively.

FIGURE 5 | Venn diagram of endophytic actinomycetes in genera level from *F. roseus* Schipcz. collected in two different geographical environments.

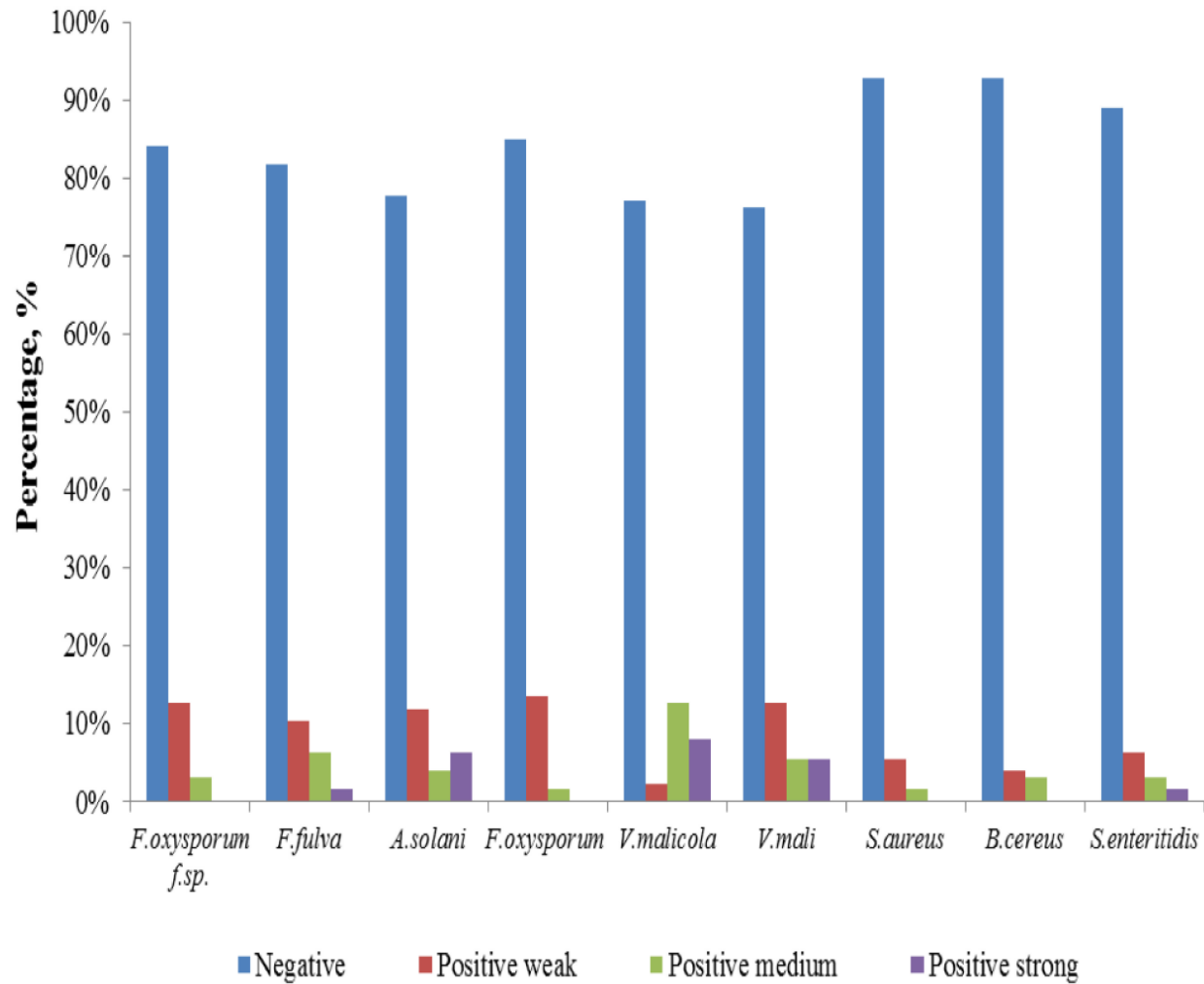
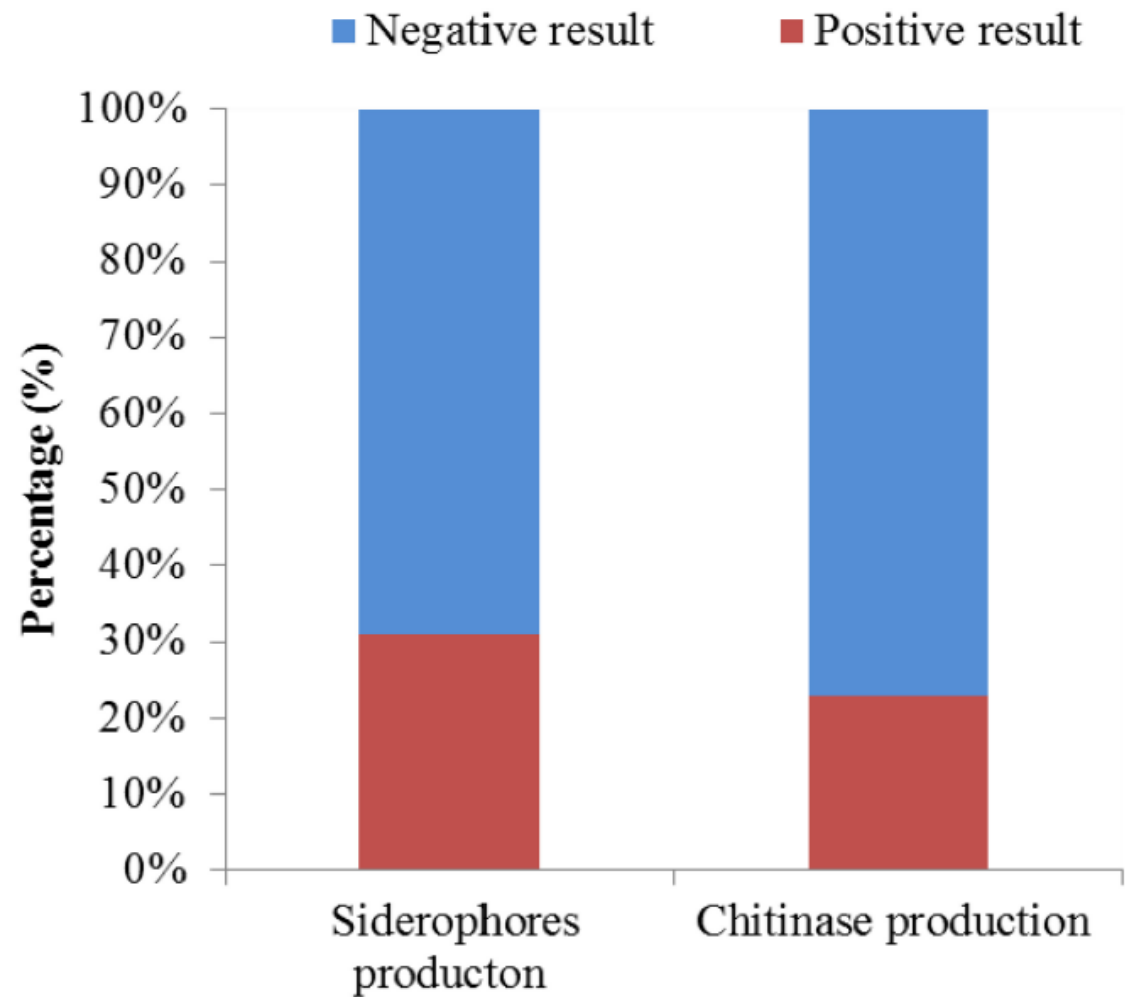


FIGURE 6 | Distribution of positive strains tested for antimicrobial potential.

## Antimicrobial Activity of the Isolates

54 strains were showed antimicrobial activity against one or more indicator pathogens.



**FIGURE 8 |** The chitinase- and siderophore-producing activities of isolates.

## Screening of Chitinase- and Siderophore- Producing Strains

**TABLE 5** | Distribution of antibiotics synthesis genes in 54 antagonistic endophytic actinomycetes.

Taxa group	Number of tested strains	Number of positive strains (%)	Positive results of the PCR test (%)		
			PKS I	PKS II	NRPS
Rare actinomycetes	21	8 (14.8)	3 (5.6)	7 (13.0)	2 (3.7)
<i>Sterptomyces</i>	33	19 (35.2)	1 (1.6)	14 (25.9)	13 (24.1)
Total	54	27 (50.0)	4 (7.4)	21 (38.9)	15 (27.8)

Antibiotics  
Biosynthetic Genes

# Discussion

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- Endophytic actinobacteria associated with medicinal plants as a biocontrol agent are considered to be beneficial for plant diseases management.
- The pretreatment of plant tissues is an advantageous method to promote and release rare endophytic actinobacteria from the inner parts of plant tissues.
- It is an essential and useful step for the isolation of endophytic actinobacteria that is depending on many factors, including host species, geographic and habitat distribution, pretreatment of plants, and selective media.

## Cont.

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- Isolates showed a great diversity in populations of endophytic actinobacteria. The diversity analysis showed that the evaluated parameters from each location sample were different.
  - In the current study, *Streptomyces*. sp. was the most dominant in the root tissues, whereas fewer species were isolated from leaves and stem. This may be associated with the fact that the rhizospheric actinobacteria can move along the internal plant tissues and can colonize the roots.

## Cont.

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- In this study, at least one antibiotic biosynthetic gene was detected in 27 (representing 50% of tested strains) antagonistic strains.
  - The absence of amplification of PKS I, PKS II, and NRPS genes in some of the tested strains may be due to the absence of these genes, and the amplified primers were not suitable.
  - This result suggested that the notion of that there is no direct correlation between the occurrence of biosynthetic genes and the production of antibacterial activities.





Thank  
you!!