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Bioactivity of endophytic bacteria and yeasts isolated from *Thymus*

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ABSTRACT

Endophytes are important resources of bioactive compounds and due to their potential for secondary metabolites production they are regarded as a potential reservoir of biotechnological applications. In this study, the bioactivity of bacteria and yeasts endophytes residing in *Thymus* sp. was evaluated. During April to October 2011, symptomless and healthy tissues of *Thymus* sp. were collected. A total of 23 strains of endophytic bacteria and 6 yeasts were isolated. The bio-effects of the endophytes were studied on *Botrytis cinerea* and plant pathogenic bacteria *Xanthomonas arboricola* pv. *juglandis* and *Streptomyces scabies* and human pathogens *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC. Growth inhibition mechanisms of the endophytes against *B. cinerea* were evaluated and it seems that the antimicrobial effects of bacterial endophytes are related to the production of protease enzyme, hydrogen cyanide and volatile compounds. Bacterial strains were identified as *Bacillus, Pseudomonas* and *Xanthomonas* of which *Bacillus* was the predominant isolate. For the first time, *Bacillus* is reported from *Thymus eriocalyx, T. lancifolius, T. fallax, T. kotschyanus* and *T. vulgaris* and *Pseudomonas* and *Xanthomonas* as endophytic bacteria from *Thymus*.

Keywords: bacteria, endophyte, *Thymus*, yeast.

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Introduction

Residing asymptomatically in plants, endophytic bacteria may play many important and beneficial roles in the metabolism and physiology of their hosts. Atmospheric nitrogen fixation (1), solubilizing of phosphates solubilizing phosphates, production or inducing phytohormones, suppressing of ethylene production, degrading toxic compounds (2), biocontrol of pathogens through direct antagonism against pathogen or the increase of systemic resistance in plants and helping in the absorption of nutrients are some of their recognized functions (3). In recent years, studies on medicinal plants endophytes have increased since it has been observed that these plants have different medicinal properties. For instance, some of them have high antimicrobial activity and in some cases, it has been found that these properties are due to the metabolites produced by the endophytes present in these plants (4-6). Since most of the secondary metabolites produced by plants are also produced by their endophytes, it is important to study endophytic population of these plants to determine their medicinal properties.

Thymus sp. is a medicinal plant that belongs to the Lamiaceae family in which its essence and extracts are used in the cosmetic, medicinal and perfume industries, as well as in food preservation. *Thymus* essence, known as Thyme essence possesses antibacterial, antifungal and antioxidant properties and delays aging in mammals and it has been officially known as an amicrobicide since the 16th century (7). In 2010, Asgharian (8) evaluated the species *T. daenensis* and could identify *Bacillus* endophytic bacteria. In 2011, Selim *et al*. (9) were able to isolate and identify endophytic *Bacillus* in the evaluation of the species *T. decussatus*.

The present research is the first study to isolate bacterial and yeast endophytes of *T. eriocalyx, T. lancifolius, T. fallax, T. kotschyanus, T. vulgaris* and *T. daenensis* (in the west of Iran) to determine their bioeffects.

Materials and Methods

Sampled sites and Host species

From April to October 2011, different healthy tissues of *Thymus* sp., were collected from natural habitats of Iran's Western parts (Table 1).

Isolation and characterization of endophytic bacteria and yeasts

Modified method of Lin *et al*. (10) was used to isolate the bacteria and yeasts from plant tissues. Plant samples were rinsed with running water to remove dust. Sterilization of plant tissues was performed using 70% ethanol and 0.5% sodium hypochlorite for 30 sec and 2 min, respectively, followed by three-times rinses in sterile distilled water. Sterilized tissues were cut into segments and placed in tubes containing 100 µl of distilled water. After 20 min, 500 µl of this solution was plated on nutrient agar medium. Plates were incubated at 28°C and observed periodically for endophytes growth. To separate yeasts and bacteria, the isolates were cultured on media containing lactic acid and tetracycline. Bacterial endophytes were characterized and identified following physio-biochemical methods according to standard protocols (11).

Table 1. Endophytic bacteria and yeasts isolated from *Thymus* species

Location (Iran)	Plant species	Plant segment	Isolate	Endophytes
Lorestan; West (33.48°N, 48.35ºE; 1907 m)	*Thymus eriocalyx*	Stem	MB1	*Pseudomonas* sp.
	T. eriocalyx	Stem	MB11	*Bacillus* sp.
	T. eriocalyx	Stem	MB13	*Bacillus* sp.
	T. eriocalyx	Stem	MB15	Yeast
	T. eriocalyx	Stem	MB45	Yeast
	T. eriocalyx	Root	MB4	*Bacillus* sp.
Markazi; Center (34.08°N, 49.70ºE; 2362 m)	*T. eriocalyx*	Leaf	MB5	*Bacillus* sp.
	T. eriocalyx	Leaf	MB10	Yeast
	T. eriocalyx	Leaf	MB46	*Bacillus* sp.
	T. eriocalyx	Stem	MB20	*Bacillus* sp.
	T. eriocalyx	Stem	MB29	*Bacillus* sp.
	T. eriocalyx	Stem	MB44	Yeast
	T. eriocalyx	Root	MB35	*Xanthomonas* sp.
	T. eriocalyx	Root	MB47	*Bacillus* sp.
Kordestan; West (35°N, 46ºE; 2045 m)	*T. kotschyanus*	Leaf	MB12	*Bacillus* sp.
	T. kotschyanus	Leaf	MB42	*Bacillus* sp.
	T. kotschyanus	Stem	MB41	*Bacillus* sp.
	T. eriocalyx	Leaf	MB36	*Bacillus* sp.
	T. eriocalyx	Stem	MB25	*Bacillus* sp.
	T. eriocalyx	Stem	MB30	*Bacillus* sp.
	T. eriocalyx	Root	MB6	*Pseudomonas* sp.
Nahavand; West (34.19°N, 48 ºE; 1644 m)	*T. lancifolius*	Leaf	MB7	*Bacillus* sp.
	T. lancifolius	Stem	MB19	Yeast
Hamedan; West (34.79°N, 48.51ºE; 1900 m)	*T. fallax*	Stem	MB16	*Bacillus* sp.
	T. fallax	Stem	MB31	Yeast
	T. daenensis	Root	MB26	*Bacillus* sp.
	T. daenensis	Root	MB27	*Bacillus* sp.
	T. daenensis	Root	MB37	*Bacillus* sp.
Hamedan; West (medicinal plants garden)	*T. vulgaris*	Leaf	MB39	*Bacillus* sp.

Pathogenic microorganisms

Pathogenic microorganisms used in this research included 2 positive gram bacteria (*Staphylococcus aureus* ATCC 33591 and *Streptomyces scabies*), 2 negative gram bacteria (*Escherichia coli* ATCC 25922 and *Xanthomonas arboricola* pv. *juglandis*) and fungus *Botrytis cinerea*. Human pathogenic bacteria were obtained from the "Collection Center of Industrial Bacteria and Fungi of Iran" and plant pathogenic bacteria from the

Bu-Ali Sina University. *B. cinerea* was received from Mirzaei *et al*. (12).

Antifungal bioactivity assay

To perform this assay, the method explained by Jalgaonwala *et al*. (13) was used, with a little modification. Suspension of 10^7 CFU/ml was provided from bacteria and yeasts endophytes. Endophytes were spot inoculated at three corners of the plates containing potato dextrose agar containing yeast extract and peptone; distilled water was used as control. 48 hours later, five-day old culture disks (5 mm diameter) of *B. cinerea* were inoculated at the center of the plates and incubated for four days at 26ºC. It should be noted that for antifungal bioactivity assay of *Bacillus* bacteria, pathogenic fungus was cultured one day ahead in plates due to the bacteria high growth rate.

The experiment was carried out in a completely randomized design in three replicates. The inhibition percentage was calculated with the following formula:

Inhibition (%) = [(growth radius in control – growth radius in treatment) /growth radius in control] × 100

The mechanisms by which endophytes affect *B. cinerea* were investigated as well. For this purpose, the production of protease (14), hydrogen cyanide (HCN) (15), cellulase (16) and volatile compounds (17) were evaluated.

Antibacterial bioactivity assay

Based on Kraus and Loper (18) modified method, the antibacterial effect of endophytes was investigated. Suspensions of endophytic isolates were provided at 10^7 CFU/ml concentration. The suspensions were properly inoculated on a nutrient agar containing yeast extract and peptone and plates were incubated for 48h at 27°C. Distilled water was used as control. The obtained colonies were cleaned from the plate's surface using sterilized cotton wool immersed in alcohol. Then, two to three drops of chloroform was added to the lid of each plate and kept upside down for 20 min. The plate's lids were opened in sterilized conditions and aerated for 30 min. Finally, one milliliter of pathogenic bacterial suspension at 10^7CFU/ml concentration was distributed on each plate. Plant and human pathogenic bacteria were incubated at 27°C and 35ºC, respectively. After 24-48h, their inhibitory effects were measured and the results were analyzed in three replicates in a completely randomized design.

Results

Thymus species and their endophytic bacteria and yeasts

Six species of *Thymus* including *T. eriocalyx*, *T. lancifolius, T. fallax*, *T. kotschyanus*, *T. vulgaris* and *T. daenensis* were obtained from which 23 bacterial and six yeast strains were isolated from their different parts (Table 1).

The bacterial strains were characterized based on standard bacteriological methods. Out of the 23 bacterial strains, 20 were characterized as gram positive and three as gram negative. Strains were identified as three genera *Bacillus*, *Pseudomonas* and *Xanthomonas* (Table 1).

Antifungal bioactivity assay

The effect of endophytic isolates on *B. cinerea* was evaluated in a completely randomized design in three replicates. There

was a significant difference between treatments at 1% level and the most inhibition percentage was observed in MB27and MB12 (*Bacillus*) by 50 and 48.03 percent respectively (Table 2). The halo was permanent at the border of endophyte and pathogen and was not covered over time which is in agreement with the results of Baghestan (19) confirming the permanency of antagonist bacterial border.

Despite that several *Bacillus* isolates affected the growth of *B. cinerea*, no remarkable effects was found for yeasts, *Pseudomonas* sp. and *Xanthomonas* sp. on this fungus (Table 2).

Table 2. Antifungal activity of endophytic bacteria and yeasts isolated from *Thymus* species on *Botrytis cinerea*

Isolate code	Isolate	Growth inhibition (%)	Isolate code	Isolate	Growth inhibition (%)
MB27	*Bacillus* sp.	50.00 a*	MB7	*Bacillus* sp.	31.37 g^{-l}
MB12	*Bacillus* sp.	48.03 ab	MB37	*Bacillus* sp.	31.37 g^{-l}
MB20	*Bacillus* sp.	44.11 bc	MB26	*Bacillus* sp.	31.37 g^{-l}
MB36	*Bacillus* sp.	41.17 cd	MB42	*Bacillus* sp.	30.39 h-m
MB30	*Bacillus* sp.	41.17 cd	MB47	*Bacillus* sp.	28.43 i-m
MB5	*Bacillus* sp.	40.19 c-e	MB45	Yeast	27.45 j-n
MB11	*Bacillus* sp.	38.23 c-f	MB1	*Pseudomonas* sp.	26.47 k-n
MB44	Yeast	37.25 d-g	MB15	Yeast	26.47 k-n
MB39	*Bacillus* sp.	36.27 d-h	MB19	Yeast	26.47 k-n
MB13	*Bacillus* sp.	36.27 d-h	MB41	*Bacillus* sp.	25.49 l-n
MB16	*Bacillus* sp.	35.29 d-h	MB46	*Bacillus* sp.	25.49 l-n
MB29	*Bacillus* sp.	34.31 e-i	MB25	*Bacillus* sp.	24.51 mn
MB4	*Bacillus* sp.	33.33 f-j	MB31	Yeast	21.56 n
MB6	*Pseudomonas* sp.	32.35 f-k	MB10	Yeast	14.70 o
MB35	*Xanthomonas* sp.	31.37 g^{-l}			

*Similar letters indicate no significant difference at 1% level.

Mechanisms of biocontrol of *B. cinerea* by endophytic bacteria and yeasts

Protease production: Regarding the formation of a clear zone around the colony of endophytic isolates 48 h after incubation, all strains were able to produce protease except *Xanthomonas.*

Production of HCN: Among the endophytic isolates, only *Pseudomonas* strains were able to produce HCN. According to Stutz *et al*.

(20), one of the most important volatile compounds produced by *Pseudomonas fluorescens* (CHA0) to control diseases is HCN. In the present research it also seems that one of the biocontrol mechanisms of *Pseudomonas* isolates is HCN production.

Cellulase production: None of the isolates could produce cellulase.

Antifungal volatile compounds: All the endophytic bacteria isolates and yeasts influenced the growth of pathogenic fungus *B.cinerea* and led to a reduction in mycelial growth. The analysis of variance showed that there was significant difference among them at 1% level. The *Bacillus* isolates MB5, MB36, MB12 and MB16 showed the greatest inhibition (Table 3).

Totally, the inhibition mechanisms of the isolates used could be due to the production of protease enzymes, HCN and volatile compounds.

Antibacterial bioactivity assay

The results showed that the yeast isolates had no effect on the tested bacteria. Of the 23 endophytic bacteria, MB20, MB27, and MB13 strains were effective against *E. coli* at 1% level of significance of which MB27 with 24.33 mm inhibitory zone was the most effective. MB20 and MB27 strains affected *S. aureus* and *X. arboricola*. MB20 with 28.33 and 15 mm zones of inhibition had the greatest effect on *S. aureus* and *X. arboricola*, respectively, while showing a significant difference with MB27 and control. None of the isolates showed any effect on *S. scabies* (Table 4). None of gram negative endophytic bacteria affected the pathogenic ones.

Table 3. The effect of volatile compounds of endophytic bacteria and yeasts isolated from *Thymus* species on *Botrytis cinerea*

Isolate code	Isolate	Growth inhibition (%)	Isolate code	Isolate	Growth inhibition (%)
MB5	*Bacillus* sp.	87.2 a*	MB30	*Bacillus* sp.	36.3 h
MB36	*Bacillus* sp.	87.2 a	MB42	*Bacillus* sp.	36.3 h
MB12	*Bacillus* sp.	87.2 a	MB1	*Pseudomonas* sp.	36.3 h
MB16	*Bacillus* sp.	87.2 a	MB6	*Pseudomonas* sp.	36.3 h
MB11	*Bacillus* sp.	85.4 b	MB4	*Bacillus* sp.	34.5 i
MB41	*Bacillus* sp.	83.6 c	MB37	*Bacillus* sp.	34.5 i
MB47	*Bacillus* sp.	45.4 d	MB45	Yeast	34.5 i
MB31	Yeast	45.4 d	MB26	*Bacillus* sp.	33.9 j
MB19	Yeast	45.4 d	MB27	*Bacillus* sp.	32.7 k
MB15	Yeast	43.6 e	MB7	*Bacillus* sp.	32.7 k
MB10	Yeast	43.6 e	MB39	*Bacillus* sp.	29.0 l
MB44	Yeast	43.0 f	MB46	*Bacillus* sp.	29.0 l
MB13	*Bacillus* sp.	41.8 g	MB25	*Bacillus* sp.	27.2 m
MB29	*Bacillus* sp.	36.3 h	MB35	*Xanthomonas* sp.	27.2 m
MB20	*Bacillus* sp.	36.3 h			

*Similar letters indicate no significant difference at 1% level.

Table 4. Antibacterial activity of endophytic bacteria isolated from *Thymus* species on plant and human pathogenic bacteria.

Isolate Code	Endophytic bacteria	Inhibition zone diameter (mm)			
		Plant pathogenic bacteria		Human pathogenic bacteria	
		Streptomyces scabies	*Xanthomonas arboricola* pv. *juglandis*	*Staphylococcus aureus* ATTCC 33591	*Escherichia coli* ATTCC 25922
MB27	*Bacillus* sp.	0	10.00 b*	25.00 b	24.33 a
MB20	*Bacillus* sp.	0	15.00 a	28.33 a	22.66 b
MB13	*Bacillus* sp.	0	0 c	0 c	20.00 c
Control	-	0	0 c	0 c	0 d

*Similar letters indicate no significant difference at 1% level.

Discussion

As demonstrated by De Siqueira *et al*. (21) the bioactive natural compounds produced by endophytes provide new options to solve the problem of drug-resistance development posed by pathogenic bacteria. Natural compounds may also be a useful source of new therapeutic agents for the effective treatment of diseases in human, plants and animals.

Thymus sp. with a long history of medicinal application has been known as a microbicide since the 6th century (7). Its antimicrobial properties can be attributed to endophytic microorganisms which reside in it. The bioeffects of *Thymus* endophytic bacteria were assessed by Asgharian (8) and Selim *et al*. (9) and *Bacillus* bacteria were isolated as endophytic bacteria. In our research, *Bacillus* had the greatest frequency among the endophytic isolates and is first reported from the following *Thymus* species; *T. eriocalyx, T. lancifolius, T. fallax, T. kotschyanus* and *T. vulgaris. Pseudomonas* and *Xanthomonas* are the first report as the endophytic bacteria from *Thymus* sp. Biological control of plant pathogens using *Bacillus* is on the increase as they can produce many different antimicrobial substances. *Bacillus* strains are capable of decomposing chitin, which is the main constituents of cell wall in many plant pathogenic fungi (22). *B. subtillis* produces an antifungal protein which exhibits inhibitory activity on mycelial growth of *B. cinerea* (23). Antifungal compounds of *Bacillus licheniformis* exhibited activity against gray mold disease of tomato and strawberry (24). The results of Wang *et al*. (25) showed that *B. coagulans* inhibited the growth of *B. cinerea* with antifungal activity higher than 80%. Our results of inhibition effects of *Bacillus* on *B. cinerea* growth are consistent with others (23-25).

The results of the present study indicate the great potential of endophytic bacteria in controlling human and plant pathogenic bacteria. Berde *et al*. (26) evaluated 37 medicinal plants to isolate endophytic bacteria and isolated 50 bacterial strains. These researchers evaluated the effect of endophytic bacteria on some human pathogenic bacteria such as *S. aureus* and *E. coli* and plant pathogenic bacteria *P. syringae* and *X. campestris*. They observed that

endophytic strains cause antibacterial activity against the mentioned pathogens. Therefore, it is suggested that endophytes can be potential resources of antimicrobial agents for biotechnological applications.

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