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Isolation and screening of phytotoxin-producing actinomycetes for biological control of *Cardaria draba*

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ABSTRACT

Cardaria draba is an important, troublesome weed in the sustainable production of wheat. In the first step of this study, 100 actinomycete isolates were purified from soil samples collected from the rhizosphere and phyllosphere of plants in different provinces of Iran. All isolates were subjected to primary screening by assaying the fermentation broth of the sterile surfaces of *C. draba* leaves. Considerable herbicidal activity was observed for four isolates. During secondary screening and assaying of the extracted culture medium with ethyl acetate, two isolates, *Streptomyces* sp. UTMC 2102 and *Streptomyces* sp. UTMC 2104, were selected as superior phytotoxin-producing isolates in the biological control of *C. draba.* Leaf necrosis was observed in 500 and 1000 µg/ml and ≥100 µg/ml dilutions for isolates UTMC 2102 and UTMC 2104, respectively. Phylogenic identification confirmed that the 16S rRNA gene has 100% similarity to *Streptomycin anulatus* for the isolate *Streptomyces* sp. UTMC 2102 and 100% similarity to *Streptomyces vinaceusdrappus* for the isolate *Streptomyces* sp. UTMC 2104. These results suggest that the *S. anulatus* and *S. vinaceusdrappus* isolates can be used in the biological control of *C. draba* in wheat fields.

Keywords: actinomycetes, biological control, Cardaria draba, phytotoxin.

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Introduction

Environmental problems, limited target sites, and the long-term toxicity of chemical herbicides on non-target organisms have become worldwide concerns in the area of weed management. Increasing demands for safe and eco-friendly alternatives to hazardous xenobiotics have motivated scientists to search for biological herbicides to protect farmlands against weeds (1, 2).

The term "biological herbicides" refers to microorganisms and/or their natural compounds which are useful in weed control (3). Microbial secondary metabolites are able to cause obvious lesions in plant tissue and are involved in plant disease are phytotoxins (1). Phytotoxins have low molecular weight, and the damage they inflict on plants includes necrosis, chlorosis, wilting, etc. There are several classifications for these natural substances; most of them are based on chemistry, organism production, biological activities, or selective toxicity to the host (4). Other than bacterial and fungal plant pathogens, several non-pathogenic soil microbes produce bioactive compounds with phytotoxic activity that can be introduced as novel templates for the herbicide industry (2). Considering the sensitivity of microorganisms to environmental conditions, the application of microbially-produced phytotoxins via fermentation is more preferable. Furthermore, phytopathogens need more time to perform in weed control than chemical herbicides or phytotoxins (5, 6).

Actinomycetes are a major group of soil inhabitant bacteria. Their unique potential for producing valuable secondary metabolites including agro-active compounds has made them a good candidate for screening projects, especially in the area of biological weed control (7).

C. draba, commonly called white top or hoary cress, is a perennial noxious weed native to Eastern Europe and western Asia, including Iran (8). The identified glucosinolate compounds in the plant root extract inhibit the germination and initial seedling growth of wheat (9).

This study aimed to isolate actinomycetes from soil of Iran and screen them for phytotoxic activity against *C. draba*.

Materials and Methods

Soil sample collection

Soil samples were collected from the rhizosphere of withered plants at a depth of 20 cm in different provinces of Iran and kept at 4°C during transportation to the lab. Each sample was air-dried at room temperature and then ground and passed through a 2 mm-mesh sieve (10).

Isolation and purification of actinomycetes

1 g of each soil sample was suspended in 100 ml physiological saline solution (NaCl 8.5 g/l) and shaken at 200 rpm at 28°C for 30 min. Serial dilutions of the suspensions of up to 10^{-6} were prepared using physiological saline. 100µl of each dilution was spread on starch-casein agar medium (11). The plates were incubated at 28°C in darkness for three weeks. The actinomycete colonies were purified on ISP2 agar plates (International Streptomyces project No. 2.) (12).

Primary screening of phytotoxic activity

Seeds of *C. draba* and Momtaz wheat cultivar (*Triticum aestivum*) were obtained from the Seed and Plant Improvement Institute, Iran. To prepare *C. draba* for bioassay, the seeds were disinfected with 5% sodium hypochlorite and placed on an MS (Murashig and Skoog) medium. The plates were preserved at room temperature until use (13, 14).

Each purified actinomycete isolate was grown in 20 ml ISP2 broth as the seeding medium and incubated at 220 rpm. After 48 h, 5% v/v of seeding materials was inoculated into 50 ml ISP2 broth as the fermentation medium and incubated at 220 rpm at 28°C for 7 days. The bacterial biomass was harvested by centrifugating the fermentation broth for 10 min (8000 ×*g*), and 40 µl of the supernatant was injected on the surface of the leaves every 24 h. This procedure was performed in triplicate. Non-inoculated ISP2 and wheat leaves were used as the negative control. Leaves were monitored for 10 days. Spore suspensions of selected phytotoxin-producing strains were kept at -20°C for further investigations.

Morphological identification of the selected isolates

After 7 days of incubation at 28°C, the cultural characteristics of the selected isolates were assessed using yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3), and inorganic salt starch agar (ISP-4) (15).

Secondary screening of phytotoxic activity

Cell-free fermentation broth of the selected strains was prepared as described and extracted in duplicate with an equal volume of ethyl acetate. The organic fractions were collected and concentrated at 37°C by rotary evaporator. The extraction process was repeated three times. Appropriate amounts of the extracts were dissolved in methanol to reach 10 mg/ml dilution as the stock vial. Dilutions of 10, 100, 200, 500, and 1000 µg/ml were prepared from the stock vial using distilled water. *C. draba* seeds were grown in 10 cm pots in a greenhouse at 27°C. Each dilution was used in assays against *C. draba* and wheat leaves both in agar plates and in pots in triplicate.

Molecular characterization of selected isolates

For genomic DNA isolation, the isolates were cultured in 15 ml Luria-Bertani (LB) broth and incubated at 28°C at 220 rpm for 48 h. DNA extraction was performed according to standard protocols (16). The 16S rDNA fragment was PCR amplified using a pair of universal primers: 9F (5'-AAGAGTTTGATCATGGCTCAG-3') and 1541R (5'- AGGAGGTGATCCAACCGCA-3') (17). The products were purified from gel agarose 1% using a Gel Extraction Kit (Qiagen, USA) and sequenced (Macrogen, South Korea). The sequences were compared with those of other validated species in the EzTaxon database (18).

Results

Isolation and purification of actinomycetes

Using the selected medium and cultivation conditions described previously, a total of 100 actinomycete colonies were purified from 18 soil samples. Most of the isolates showed moderate growth on ISP2 agar. All 100 isolates were preserved in the University of Tehran Microorganisms Collection (UTMC).

Primary screening of phytotoxic activity

All 100 isolates were screened for phytotoxic activity against *C. draba* and wheat leaves, but only four isolates, UTMC 2101, UTMC 2102, UTMC 2103, and UTMC 2104, showed herbicidal activity on *C. draba* in their fermentation broth 24 h after the first injection. The wheat leaves remained intact. No lesions were observed in *C. draba* leaves from the other isolates or the non-inoculated ISP2 until ten days had passed. The purified colonies of the four mentioned isolates are presented in Figure 1.

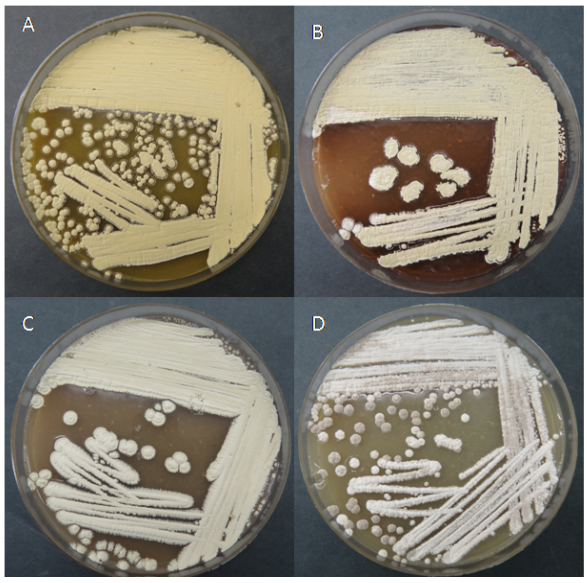

Figure 1. Purified colonies of the four selected isolates during the primary screening on ISP2 agar. (A) Isolate UTMC 2101. (B) Isolate UTMC 2102. (C) Isolate UTMC 2103. (D) Isolate UTMC 2104.

In the present study, we classified the isolates according to their phytotoxic activity (2) against *C. draba* into four categories of no (-), weak (+), moderate (++), or strong (+++) activity (Table 1). Some morphological and cultural characteristics of these four phytotoxic isolates, such as the color of the aerial mycelium and the color of the soluble pigment on ISP2, ISP3, and ISP4 media, are summarized in Table 2. As can be seen, none of the isolates produced soluble pigment in the ISP3 medium.

Herbicidal assay of the extracts

Biological assays of the dilution prepared from the cell-free fermentation broth of isolates UTMC2102 and UTMC2104 showed necrosis and chlorosis on *C. draba* leaves at 500 and 1000 µg/ml dilutions and >100µg/ml dilutions, respectively. The results were the same in both agar plate and pot trials; however, as with all dilutions of the non-inoculated ISP2 extract, no lesions were observed in all dilutions of fermentation broth of strains UTMC 2101 and UTMC 2103. Wheat leaves remained intact after all experiments. Necrosis of *C. draba* leaves in agar plates and in pots are presented in Figures 2 and 3, respectively.

Comparison of 16S rRNA gene of strains UTMC 2102 and UTMC 2104 with other related strains

The two selected phytotoxic isolates obtained in this research were identified based on partial sequencing of 16S rDNA. The obtained nucleotide sequence data have been deposited at GenBank under accession numbers KF 381400 and KF381401. The similarity percentages were 100% (Table 3). Based on the BLAST results, isolates UTMC 2102 and UTMC 2104 belong to *S. anulatus* and *S. vinaceusdrappus*, respectively.

Table 1. Phytotoxic activities of actinomycetes isolates against *C. draba*.

Isolate	Phytotoxic activity*	Isolate	Phytotoxic activity
UTMC 2101	+++	UTMC 2104	+++
UTMC 2102	++	UTMC 2105-UTMC 2100	-
UTMC 2103	+	Non-inoculated ISP2	-

* Based on their phytotoxic activity; isolates were classified into four categories of no (-), weak (+), moderate (++) or strong (+++) activity.

Table 2. Cultural characteristics of the four strains with phytotoxic activity on different ISP media.

Isolate No.	Yeast extract-malt extract (ISP2) medium			
	Color of aerial mycelium	Color of reverse side of colony	Color of soluble pigment	Growth
UTMC 2101	Yellow	Light brown	Light yellow	Heavy
UTMC 2102	Yellowish white	Dark brown	brown	Moderate
UTMC 2103	White	Light brown	-	Moderate
UTMC 2104	Grayish white	cream	-	Heavy
	Oatmeal (ISP3) medium			
	Color of aerial mycelium	Color of reverse side of colony	Color of soluble pigment	Growth
UTMC 2101	Light yellow	Yellow	-	Moderate
UTMC 2102		Light brown	-	Heavy
UTMC 2103	Yellowish white	Cream	-	Heavy
UTMC 2104	Grey	cream	-	Heavy
	Inorganic salts-starch iron (ISP4) medium			
	Color of aerial mycelium	Color of reverse side of colony	Color of soluble pigment	Growth
UTMC 2101	Dark yellow	Orange	orange	Moderate
UTMC 2102	Cream	Cream	-	Moderate
UTMC 2103	White	Cream	-	Moderate
UTMC 2104	Grayish white	Cream	-	Moderate

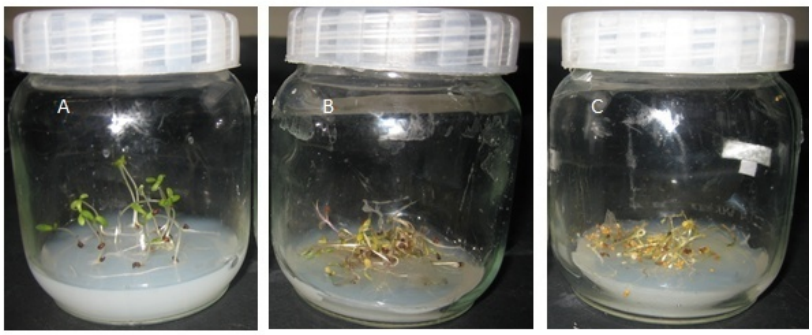

Figure 2. *C. draba* in MS medium one week after injection of extracts. (A) After injection the 500 µg/ml dilution of non-inoculated ISP2 extract (control). (B) After injection the 500 µg/ml dilution of the extract of the isolate UTMC 2102. (C) After injection the 500 µg/ml dilution of the extract of the isolate UTMC 2104.

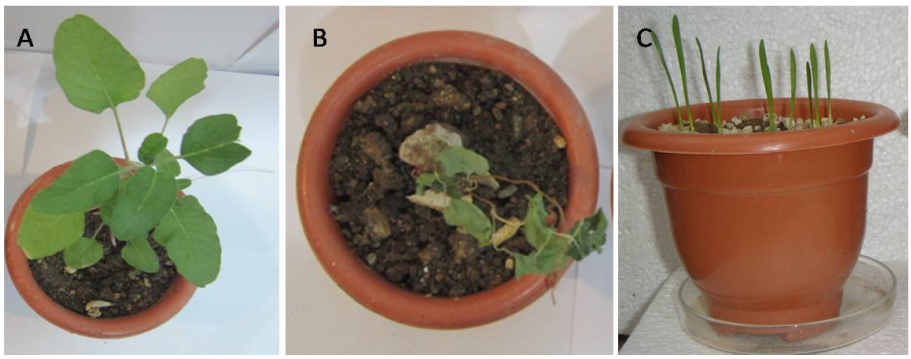

Figure 3. ***C. draba*** **and wheat in pots one week after injection of extracts. (A)** ***C. draba*** **after injection the 500 µg/ml dilution of non-inoculated ISP2 extract (control). (B)** ***C. draba*** **after injection the 500 µg/ml dilution of the extract of the isolate UTMC 2102. (C) wheat after injection the 500 µg/ml dilution of the extract of the isolate UTMC 2102.**

Table 3. Identification of phytotoxin producing isolates by partial sequencing of 16S rDNA gene

Isolate	GenBank accession No.	The most similar strain	Accession number	Similarity %
Streptomyces sp. UTMC 2102	KF381400	*Streptomyces anulatus* NRRL B-2000(T)	DQ026637	100
Streptomyces sp. UTMC 2104	KF381401	*Streptomyces vinaceusdrappus* NRRL 2363(T)	AY999929	100

Discussion

80% of the total 8000 discovered bioactive metabolites in actinomycetes belong to the genus *Streptomyces,* and over 150,000 bioactive metabolites are still waiting to be identified (19). *Streptomyces* has a significant capacity to produce important agro-active compounds for commercial use. Phosphinothricin from *S. viridochromogenes* and *S. hygroscopicus* and Bialaphos from several *Streptomyces* species are two well-known *Streptomyces* phytotoxins (2).

Molecular characterization techniques, such as 16S rRNA gene sequence analysis, are more suitable and time-saving approaches to identifying *Streptomyces* compared with classical identification methods which are based on phenotypic characterizations (20). The production of agro-active compounds for *S. anulatus* has not been reported. This paper is the first to report on the production of metabolites for crop protection and herbicidal activity for this strain. Recently, the presence of an antiviral substance against cucumber moasic virus has been seen in culture filtrates of the strain *S. vinaceusdrappus* (21). This compound is not a phytotoxin, but the strain can be regarded as valuable bacteria in the biological control of pests and plant diseases.

Using surface sterilized plant seeds in agar plates is a convenient approach for screening microbial isolates for phytotoxic activity. Since most phytotoxins are hydrophilic exotoxins, the broth culture after centrifugation or filtration contains the bioactive component. Therefore, observing herbicidal activity in cell-free broth guarantees the ability of the isolates to produce phytotoxic compounds in a submerged culture rather than on an agar medium. It is important to consider that the concentration of phytotoxins in the broth depends greatly on the broth

formulation, and in most cases, it is very low. Therefore, as saying the broth extracts for phytotoxicity can aid in the selection of efficient isolates. Water-immiscible organic solvents such as ethyl acetate, chloroform, dichloromethane, and ether are suggested for the removal of phytotoxins from the broth, because most of them are soluble in these solvents (22).

Since no approved biocontrol agent is available for *C. draba*, the obtained data of the present study can be a prerequisite for developing an appropriate agent for biological control of *C. draba*.

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