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Overview of *Pestalotiopsis vismiae* UTMC 5019 as a Potential Agent for the Biological Control of *Hordeum spontaneum* (Wild Barley)

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ABSTRACT

Hordeum spontaneum, which is one of the most important and troublesome annual weeds in crop fields around the world, causes extensive losses every year. The purpose of this study was to screen phytopathogenic fungi for biological control of this weed. For this purpose, firstly, 150 fungi were isolated from different soils in Iran. Thirty fungal strains were selected by consideration of their herbicidal activities against mature wild barley leaves. Common wheat was used as a negative control in all experiments. Finally, 6 strains showed necrosis lesions on wild barley leaves without any effects on wheat (control). The best result was achieved by Hcr99 isolate. Based on microscopic and Internal transcribed spacer-PCR (ITS-PCR) analysis, Hcr99 displayed 100% phylogenetically similarity to *Pestalotiopsis vismiae* (*P. vismiae*). 1×10^7 conidia/ml of *P. vismiae* showed the most suppression effect on weed seeds germination and rhizoid extension. Inoculum concentration of 1×10^6 and 1×10^7 conidia/ml provided significant results of weed controlling in greenhouse experiments. Host range study indicated that *P. vismiae* could induce disease symptoms in 4 weed plant species out of a total of 24 species belonging to 9 families.

Key words: Biological control, Pestalotiopsis vismiae, Bioherbicide, Wild barley, Phytopathogenic fungi.

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1. INTRODUCTION

Wild barley, (*Hordeum spontaneum* K.) is one of the most important weeds growing in wheat and crop fields. It is an annual weed which belongs to *Poaceae* family (1, 2). This weed has been frequently seen in west Asia region, especially in south and south-western parts of Iran. Indeed, today it exists in more than 16 provinces of Iran and considered as a potent threat to wheat production (3, 4). Wild barley can grow faster than wheat and has a great ability to resist against environmental stress. It is considered as an important weed and can decrease the production of wheat up to 80 % (5, 6). Today, chemical herbicides and mechanical methods are common approaches to wild barley management. Chemical herbicides are generally not specific and effective in controlling this weed (7, 8). In addition, the excessive use of chemical herbicides has negative effects on both human health and environment and is related to the expansion of herbicide resistance among weeds (9). Mechanical removing and crop rotation are the most successful methods for controlling wild barley (10). In recent decades, biological control of pest has seemed to be an appropriate choice to controlling weeds which has some advantages such as specificity to weeds, the quality of being environment-friendly and inhibition of the growth in resistance among weeds (11). Typically, in different studies, nematodes, insects, bacteria and fungi are used as bioherbicide agents, but fungi fascinate the most attention because of their high potential to make disease in plants (12, 13). In fact, fungal plant pathogens have been evaluated as potential bioherbicides for weed control since 1970s (14). Boyetchko et al (1997) used *Puccinia canaliculata* to control yellow nutsedge (*Cyperus*

esculentus L.) in all cropping areas. It has been proved that this component could completely inhibit weed flowering in early spring and reduce yellow nutsedge stand density and new tuber formation by 46 % and 66 %, respectively. Another research has proved that *Colletotrichum gloeosporioides* can control northern jointvetch (*Aeschynomene virginica* L.), a leguminous weed in rice (*Oryza sativa* L.) and soybeans (*Glycine max* L.). Its registered product which is known as ''Collego'' can be applied into the foliage with conventional herbicide sprayers in order to control this weed (15, 16). The aim of this study was to screen phytopathogenic fungi from two provinces of Iran and determine their host range and aggressiveness toward different weed and crop plants in order to introduce potent strain for biological control of wild barley. This research was the first attempt to controlling this weed by fungi around the world.

2. MATERIALS AND METHODS

2.1. Plant seeds

The seeds of wild barley (*Hordeum spontaneum* K.) and wheat (*Triticum aestivum* L.) were prepared from The Iranian Research Institute of Plant Protection, Tehran, Iran.

2.2. Culture media

Potato dextrose broth (PDB) (Sigma-Aldrich) was used as seeding and fermentation media. PDB plus 15 g/L agar was used for the isolation of fungi and sporulation. Water-agar medium (15 g agar and distilled water to make 1 L) was used for weed seeds germination and rhizoid extension (17). The sterilized seeds of Wild barely were cultivated in Murashige and Skoog medium (MS) in a growth chamber with controlled temperature (28 ^{o}C) (18).

2.3. Collection of plant material and soil sample

For the isolation of fungal species, damaged leaves, stems, roots and rhizospheric soil samples were collected from randomly selected wild plants from different provinces of Iran (states of Tehran and Khuzestan). Samples were placed in clean plastic bags, brought to the laboratory and used for further experimental purpose (19).

2.4. Isolation and purification of fungi

Collected leaves, stems, and roots were washed under slow running tap water for 15min in the laminar air flow cabinet. After proper drying of surface sterilized plant material, leaves, stems and roots were cut into pieces and each piece was placed on potato dextrose agar (PDA) medium. Besides, the suspensions of soil samples were cultured on PDA as well. Plates with plant tissues and suspension of soil samples were sealed using parafilm tape and incubated at 28±2°C in order to recover the various colonies of fungi. The observation was made for 3 days. At the end, all selected isolates were subcultured in PDA medium and finally, were maintained at 4°C till further used (19).

2.5. Primary screening of fungal isolates

The surface of wild barley seeds were disinfected with sodium hypochlorite 1 % and were placed on water-agar and MS agar as the growth medium, respectively (20). The plates were preserved at room temperature for 120 h and 168 h, respectively. The young leaves were used for bioassay. Spore suspension (1×10^7 per ml) of the fungi was prepared in phosphate buffer at pH 7 (21). Conidial suspension (1×10^7 per ml) was sprayed on sterile surface of wild barley. Fungal strains with more than 40 % damage on wild barley leaves were selected for further study. The cultivated plants were kept in dew period (RH=100 %, 28 °C and complete darkness) for 20 h, then, they were incubated in greenhouse condition and monitored daily for 3 weeks.

2.6. Secondary screening of fungal isolates

For pot test as secondary screening, 4-5 seeds of wild barley were planted in 10 cm^3 pots. The pots were kept on a greenhouse condition and after 6-8 weeks, the leaves were treated with conidial suspension (1×10^7 per ml). All assays were performed in triplicates in two independent runs. The selected fungal strains were preserved in University of Tehran Microorganisms Collection (UTMC). For optimizing spore concentrations, the plants were inoculated to complete wetness with an aerosol sprayer and then incubated in a dew chamber set at 100 % relative humidity (RH) for 20 h at 28 °C in darkness. Subsequently, the pots were transferred to greenhouse condition. Control plants were treated similarly except that they were sprayed with uncultured PDB and sterile distilled water. Wheat in sterile and pot cultures was used as a control plant in all biological assay tests. Disease symptoms and its severity for each plant in the experimental plan were categorized using a rating procedure based on the damage square with the scale of 0 to 4, where 0: no visible symptoms; 1: 0-25 %; 2: 25-50 %; 3: 50-75 %; 4: more than 75 % of the plant leaves were injured (21). Disease harshness or pathogenicity percent was measured 21 days after the inoculation.

2.7. Seed germination and root elongation inhibition test

For seed germination inhibition test, 50 wild barley seeds were placed in a Water Agar medium and conidial suspension (~1×10^7 per ml) of the selected strain(s) was sprayed on the seeds and the plates were incubated at 28 °C in darkness. Seed germination was monitored for 6 days. For root emersion, conidial suspension (~1×10^7 per ml) of the selected strains was sprayed on 50 germinated seeds which were placed in a Water Agar medium and the ability of fungal strain(s) in prevention of root emersion was monitored. Then, the plates were incubated at 28 °C in the darkness like the previous part. These experiments were repeated 3 times. Wheat was used as a control plant as well.

2.8. Optimization of inoculum spore suspension on disease

severity

Wild barely plants were sprayed with a conidial suspension containing 1×10^4; 1×10^5; 1×10^6 and 1×10^7 spore/ml. The pots sprayed with distilled water attended as controls. The plants were inoculated and subjected to a dew period as described already. Disease severity was evaluated after 21 days on the disease severity scale described. The experiment was set with three replicates in two different runs.

2.9. Study on the host range specific activity and pathogenicity of the selected isolate(s)

Host specific activity and pathogenicity of the selected isolate(s) were evaluated to 24 plant species belonging to 9 different families according to the testing strategy proposed by Wapshere (1974) (22). Triplicates of each plant were sprayed with 1 ml of 1×10^7 spore suspension. Wild barley and wheat were sprayed as positive and negative controls, respectively. After inoculation, all studied plants were kept in the dew chamber (100% RH, 28 ℃, and total darkness) for 20 h for spore germination. The plants were considered either immune (no visible reaction), resistant, (nonspreading <1 mm diameter necrotic spots), or susceptible (spreading >2 mm diameter necrotic spots) by visual observation 3 weeks after inoculation (21).

2.10. Molecular identification of selected isolate

DNA extraction was accomplished with cell lyses by liquid nitrogen and phenol-chloroform method (23). PCR was done with ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (24). The PCR program was as follows: DNA template was denatured for 5 min at 95 °C and then was amplified for 30 cycles of 30 s at 95 °C, 1 min at 54 °C and 1 min at 72 °C. The final extension time was 72 °C for 5 min. PCR products were detected by 1 % agarose gel electrophoresis and ethidium bromide staining and then were purified from gel agarose using Gel Extraction Kit (Qiagen, USA) and sequenced (Macrogen Co., Korea). The sequences were compared with those of other validated species in NCBI database.

2.11. Phylogenetic analysis

ITS region sequence of 16 isolates and sequences available at NCBI database were compared for biodiversity and systematic situation. The sequences were aligned with ClustalX2 followed by an edition in Bioedit Software and construction of neighbor-joining phylogenetic tree using MEGA 5.01 software at 500 bootstraps (25).

2.12. Statistical analysis

The SPSS statistical software package version 21.0 was used to run the T-test used for data analysis. Normality of conidial suspensions was tested using Kolmogorov-Smirnov test (KS-test).

3. RESULTS AND DISCUSSION

3.1. Primary and secondary screening

From 25 soil samples were collected from rhizosphere and phyllosphere of withered plants in different provinces of Iran, 150 fungal isolates were obtained. Spraying of spore suspension of 150 fungal strains on wild barley leaves revealed that 6 isolates made a disease symptom and necrosis effect on tested weed leaves at the end of 3 weeks. According to disease severity, onset of disease symptoms and no activity on wheat as a negative control plant, Hcr99 isolate was selected as the best candidate for controlling wild barley (Figure 1).

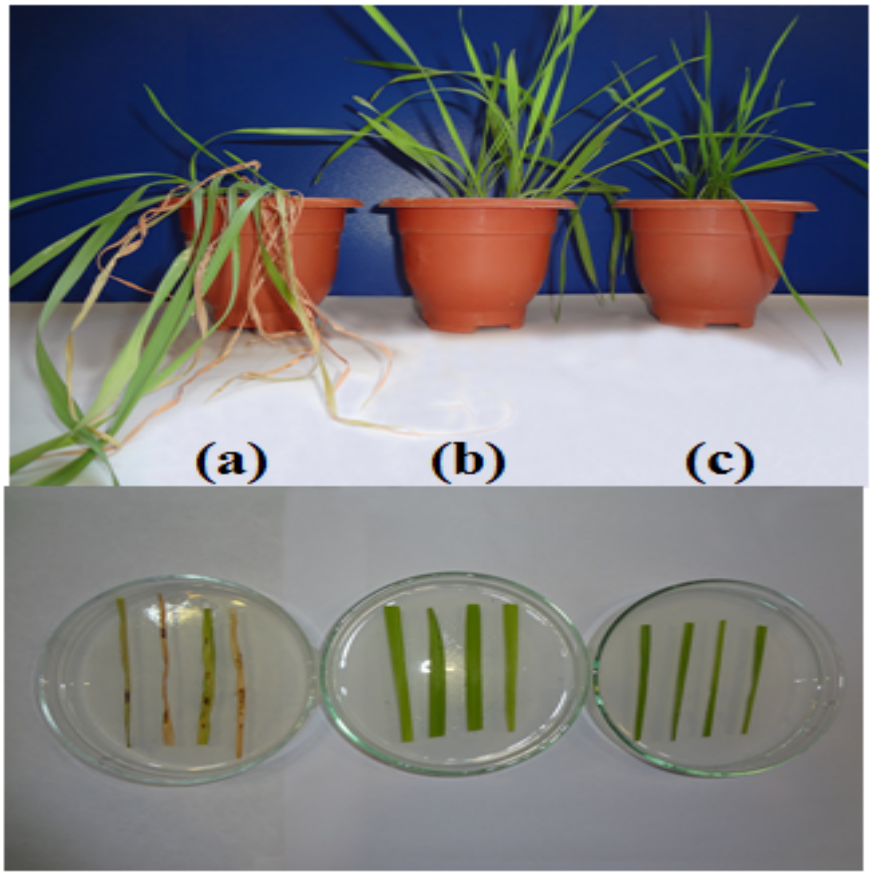

Figure 1. a) Inoculated *H. spontaneun* with 1×10^7 conidia/ml of Hcr99 isolate; b) *H. spontaneun* inoculated with distilled water as control; c) Inoculated common wheat with 1×10^7 conidia/ml of Hcr99 isolate as control

3.2. Morphological and molecular identification of selected isolate

Macroscopic and microscopic images of isolate Hcr99 have been shown in Figure 2.

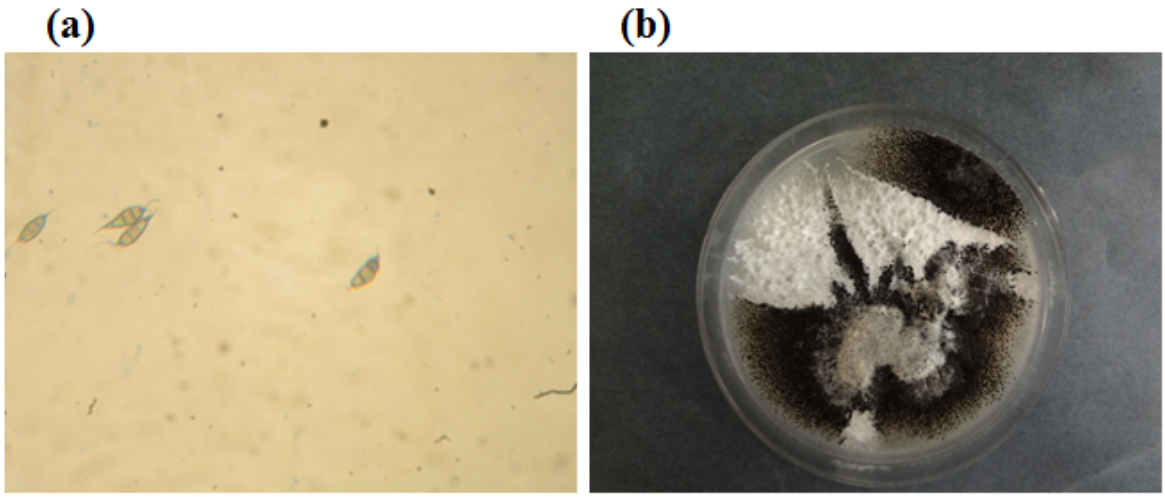

Figure 2. *P.vismiae* UTMC 5019: a: Colony in PDA and b: Lactophenol cotton blue staining of conidia

The used primers in this study (ITS1 and ITS4) were successfully amplified DNA from intended fungal strain. At the end, a product of approximately 700 bp was obtained (Figure 3).

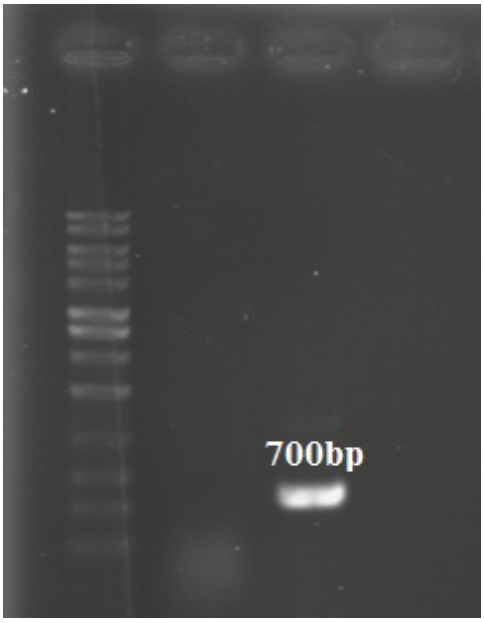

Figure 3. Amplified DNA in the ITS region from the isolated fungus using universal ITS primers

Based on sequencing results with ITS primers, it was found that the Hcr99 isolate was phylogenetically identical to *Pestalotiopsis vismiae* with the 100 % similarity and preserved in University of Tehran Microorganisms Collection (UTMC) under accession number UTMC 5019. Furthermore, phylogenetic tree of *P. vismiae* UTMC 5019 with 16 related species was shown via a neighbor-joining tree in Figure 4.

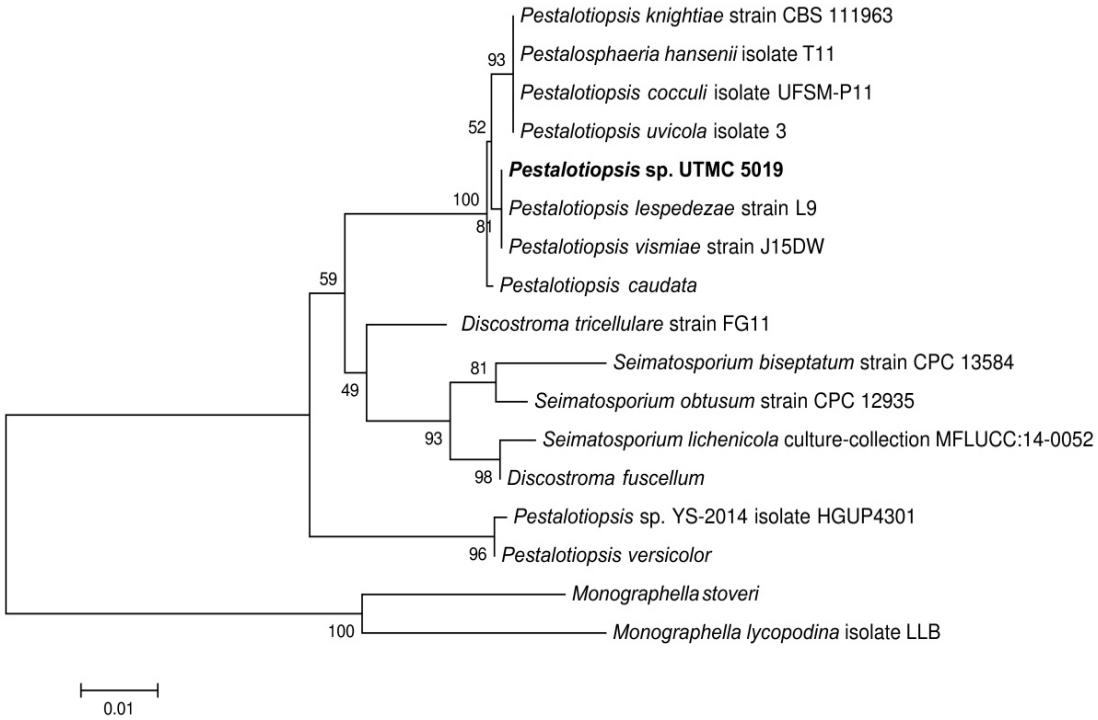

Figure 4. Neighbor joining Phylogenetic tree derived from the internal transcribed spacer (ITS) region of *P. vismiae* UTMC 5019 and 16 related species using ClustalX2, Bioedit and Mega 5.01 Software at 500 bootstraps

3.3. Suppression of seed germination and root elongation of H. spontaneum by P. vismiae UTMC 5019

Seed germination inhibition was observed 6 days after spraying ~1×10^7 spore/ml. The results indicated that *P. vismiae* UTMC 5019 could reduce the potential of seeds germination from 90 % to less than 30 %. Moreover, this isolate showed high ability to decrease the root emersion. It was found that *P. vismiae* UTMC 5019 conidial suspension had decreased the length of root from 65 mm to 25 mm (Figure 5).

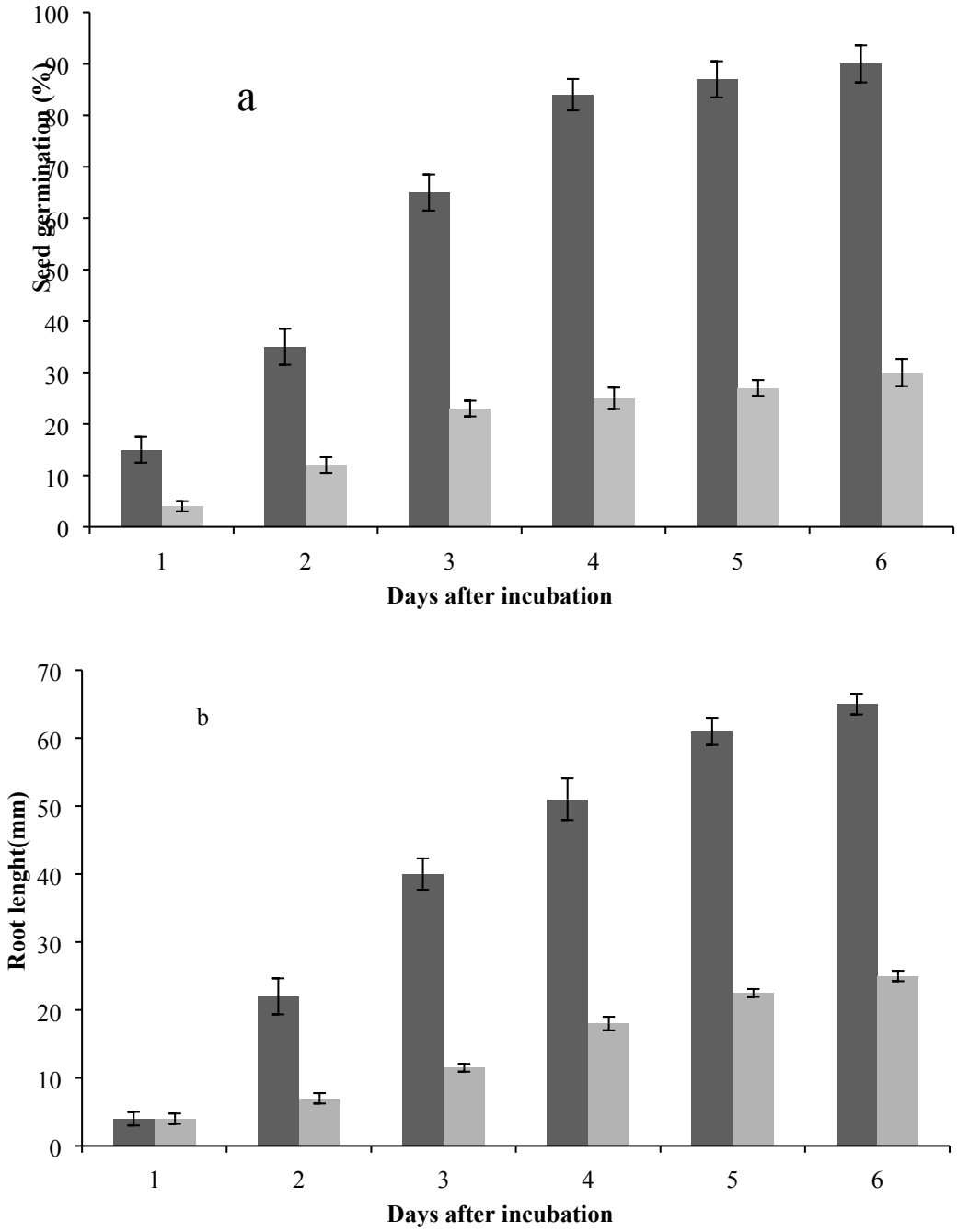

Figure 5. a) Effect of *P. vismiae* UTMC 5019 on the potential of wild barely seeds germination. The black columns represented non-inoculated seeds and the grey columns represented inoculated seeds; b) Effect of *P. vismiae* UTMC 5019 conidial suspension on *H. spontaneum* root emersion. Black columns represent the negative control and the white ones represent the treated plants. The mean difference is significant at P<0.05

3.4. Effect of P. vismiae UTMC 5019 spore count on disease severity on wild barely

The minimum spore concentration for whole plant death was measured ≥1×10^6 after 21 days. The first symptoms appeared 72 h after incubation and produced numerous small spots over the leaf surface, extensive blighting and necrosis of tissues and death of the entire leaf. In lower spore concentration, plant damage was not complete and in higher spore concentration, the results were similar with that of 1×10^6 spore/ml (Figure 6).

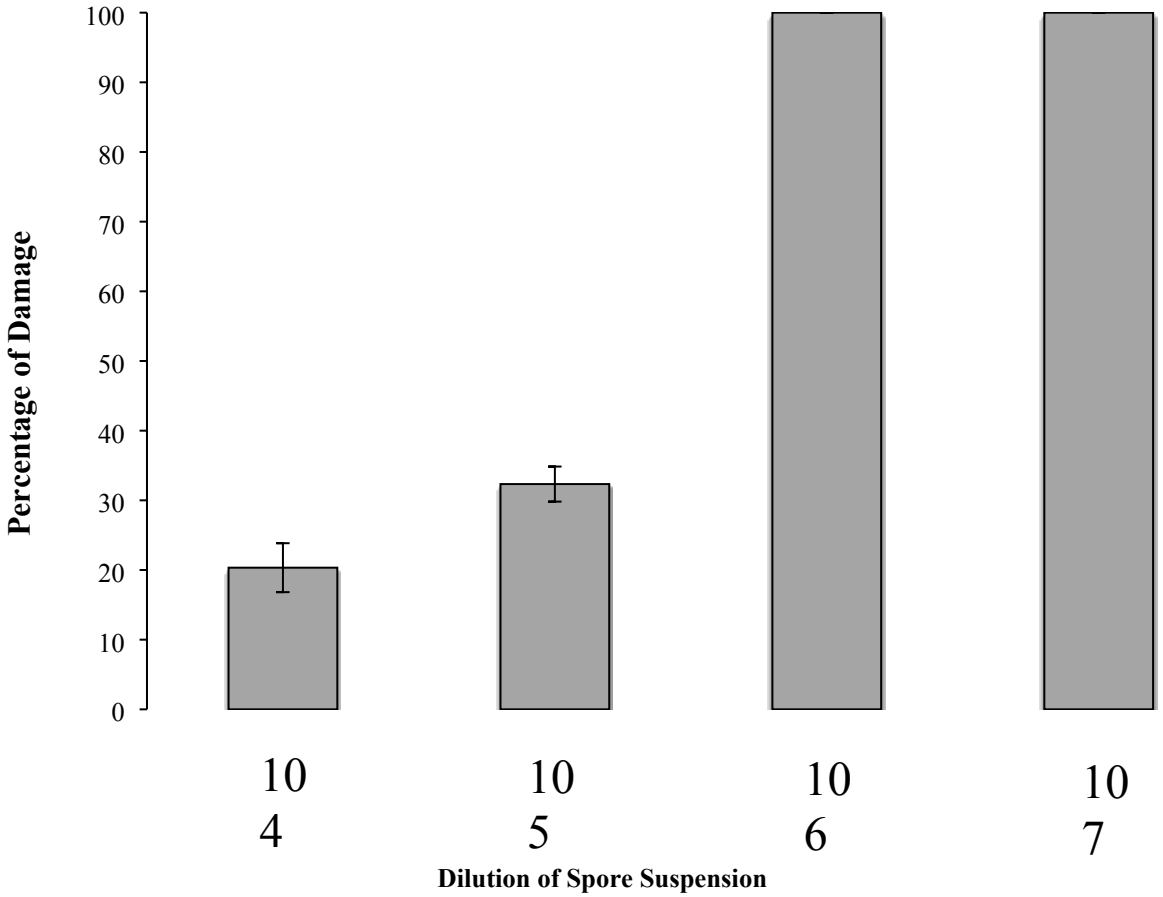

Figure 6. Potential of *P. vismiae* UTMC 5019 different conidial suspension on *H. spontaneum* after 21 days. The columns represented the plants which sprayed by 1×10^4, 1×10^5, 1×10^6 and 1×10^7 conidial per ml of selected isolate, respectively. The mean difference is significant at $P<0.05$. Vertical bars represent standard error of mean

3.5. Host range study

In the host range study, 4 weed plants out of totally 24 examined plant species developed disease symptoms (Table 1).

Table 1. Host range of *P. vismiae* UTMC 5019 for different weed (W) and crop (C) plants. The plants were considered either immune (I), resistant (R) or susceptible (S) by visual observation 3 weeks after inoculation

Disease reaction	*Type of plant*	*Common name*	*Plant species*
I	W	Redroot pigweed	*Amaranthus retroflexus* L.
I	W	Prostrate pigweed	*Amaranthus blitoides* S.
I	W		*Brassica deflexa* Boiss.
I	W	Goosefoot	*Chenopodium album* L.
S	W	Bindweed	*Convolvulus arvensis* L.
R	W	Strangleweed	*Cuscuta Pentagona* L.
I	W	White clover	*Trifolium repens* L.
I	W	Cockspur grass	*Echinochloa crus-gali* L.
S	W	Wheatgrass	*Eremopyrum bonaepartis S.*
R	W	False barley	*Hordeum murinum* L.
S	W	Wild barely	*Hordeum spontanum* K.
I	W	Ryegrass	*Lolium multiflorum* Lam.
S	W	Johnson grass	*Sorghum halepense* L.
I	W	*Yellow dock*	*Rumex crispus* L.
I	C	Wheat	*Triticum aestivum* L.
I	C	Leek	*Allium ampeloprasum* L.
I	C	Radish	*Raphanus sativus* L.
I	C	Watermelon	*Citrullus vulgaris* Thunb.
I	C	Pumpkin	*Cucurbita moschata* Duc.
I	C	Pea bean	*Phaseolus vulgaris* L.
I	C	Pea	*Pisum sativum* L.
I	C	Lentil	*Lens culinaris* Medik.
I	C	Rice	*Oryza sativa* L.
I	C	Maize	*Zea mays* L.

None of the control plants showed any disease. Three members of the *Poaceae*, including *H. spontaneum*, *Eremopyrum bonaepartis* and *Sorghum halepense* and one member of *Convolvulaceae, Convolvulus arvensis,* developed disease symptoms (Figure 7).

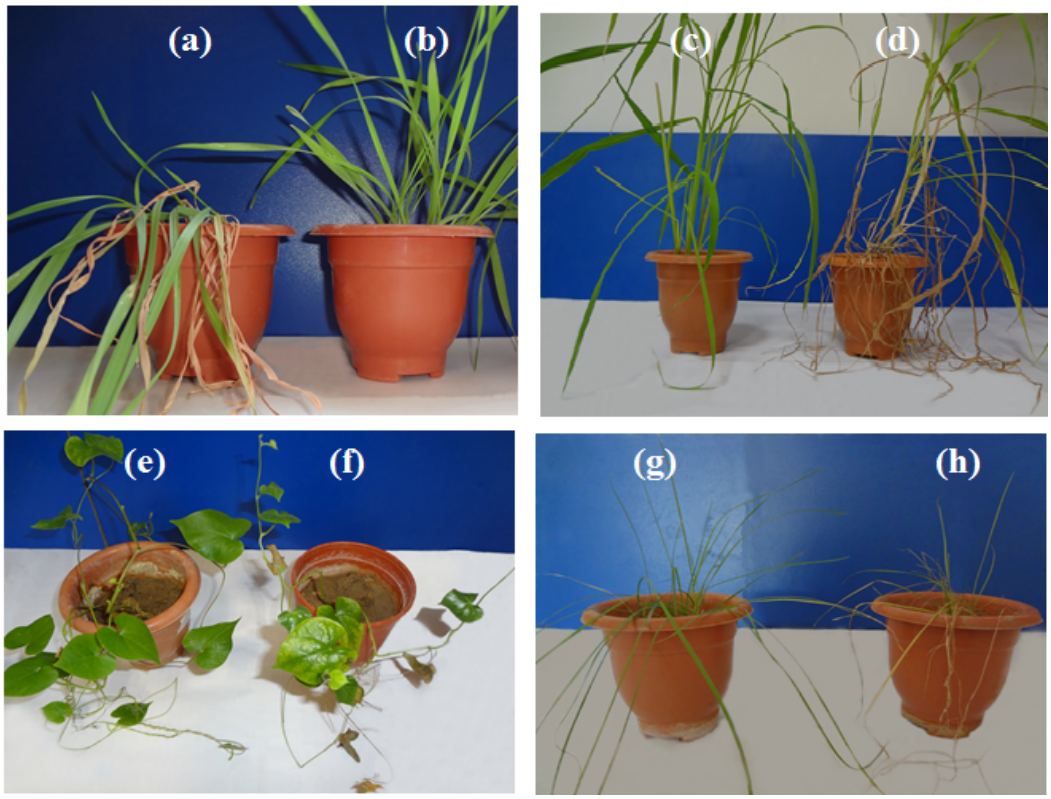

Figure 7. Effects of *P. vismiae* UTMC 5019(1×10^7 per ml) on sensitive weed plants. a) fungi spore treated; b) phosphate buffer treated *H. spontanum*; c) fungi spore treated; d) phosphate buffer treated *S. halepense*; e) fungi spore treated; f) phosphate buffer treated *C. arvensis*; g) fungi spore treated and h) phosphate buffer treated *E. bonaepartis*

As mentioned in the literature review, wild barely is one of the major problematic weeds in the crop production fields, particularly in Iran that makes a considerable loss each year. It was indicated that wild barely has the potential to make more than 70% loss in wheat production (26). Today, in order to counter this weed, Sulfosulfuron (Apiros®) and Metsulfuron methyl + Sulfosulfuron (Total®) are widely used (27, 28). The high persistence of these compounds in soil and also the toxicity associated with their application are the major disadvantages of these chemical herbicides (29, 30). In biocontrol of weeds, agents like fungi are economically efficient and have the potential to obliterate these defects as well. *P. vismiae* belongs to Amphisphaeriaceae family. Some species of this family, such as *Coryneopsis rubi* and *Pestalotia* sp, are highly potential candidates for biological control of weeds (31). One interesting finding is introducing members of *Pestalotiopsis* genus as biological agent of weed control. Members of *Pestalotiopsis* are common in tropical and temperate ecosystems (32). *Pestalotiopsis* sp. contains more than 200 species some of which cause diseases on a variety of plants (33, 34). Besides, some of *Pestalotiopsis* species are of great importance due to their valuable secondary metabolites such as anti-cancer drug taxol, jesterone, ambuic acid, torreyanic acid, pestaloside, pestalotiopsins and 2-a hydroxydimeniol that have been obtained from *P. microsporea* (35-37). The current study revealed that *Pestalotiopsis* species could have considerable potential in biological control of weeds. Also, Integration of *P. vismiae* UTMC 5019 conidial suspension with low dose of chemical herbicides like Total® and Apiros® ought to be tested due to the possibility of synergistic relation between them. *P. vismiae* UTMC 5019 showed a considerable potential to control seed germination and root emersion of *H. spontaneum*. Nevertheless, in order to ensure these abilities, it is suggested that these tests should be examined at field trial under different conditions. Superisingly, it was found that the host range data represented that *P. vismiae* UTMC 5019 is relatively host specific at the familial level. The disease symptoms were observed on the three members of Poaceae, including *H. spontaneum*, *Eremopyrum bonaepartis* and *Sorghum halepense* and *Convolvulus arvensis* that was a member of *Convolvulaceae*. These results are in agreement with those obtained by Boyetchko (16), that under controlled condition, concentrations of 10^6 and 10^7 of conidial suspensions per ml of mycoherbicides were sufficient to kill the weed after 21 days. It can therefore be assumed that *P. vismiae* UTMC 5019 shows high ability to be a suitable mycoherbicide to control *H. spontaneum*. This study was the first report on bioherbicide activity of *P. vismiae* isolate against wheat field weeds like *H. spontaneum*.

4. CONCLUSION

Plant pathogens have been used safely and effectively as registered bioherbicides to manage several weeds. Efforts are continuing in Iran to develop and register bioherbicides targeting wild barely. In addition, this research is in progress to develop an integrated management system for controlling this troublesome weed.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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