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Biocontrol activity of endophytic fungus of barley, *Microdochium bolleyi*, against *Gaeumannomyces graminis* var. *tritici*

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Abstract: In this study, two isolates of genus *Microdochium* (W2 and B26) was isolated from the roots of healthy barley plants in agricultural fields from Kermanshah province in 2014 and were identified as *Microdochium bolleyi* based on morphological and molecular characteristics. Dual culture studies revealed that W2 and B26 inhibit 45% and 20% of the radial growth of *Ggt* in turn. The W2 isolate was inoculated on barley roots in order to assess its effect on suppressing take–all disease and promoting the growth of barley plants. Regarding suppression of disease test, pathogenicity index (the percentage of necrosis root disease severity) for the plants that were inoculated with endophytic *M. bolleyi* and *Ggt* at the same time was 0.6, compared to 4.4 for the plants that were inoculated with *Ggt* alone. *M. bolleyi* also increased significantly root fresh weight by 31.21%, aerial fresh weight by 15.15%, root length by 3.0%, aerial length by 2.35%, root dry weight by 30.94% and aerial dry weight by 12.28% which were significant differences at the 5% level. For growth–promoting effects, growth parameters were evaluated and the results showed *M. bolleyi* effectively promoted root fresh weight by 60.0%, aerial fresh weight by 38.46%, root length by 4.54%, aerial length by 7.21%, root dry weight by 60.43% and aerial dry weight by 38.60%, which were significant in 5% level. To our knowledge, this is the first report of *M. bolleyi* for the mycobiota of Iran and it may be further used as a biocontrol agent.

Key words: Biocontrol agent, plant growth promotion, take–all

INTRODUCTION

Barley (*Hordeum vulgare* L.) is ranked as the fourth most important cereal crop in the world. It can also be grown profitably on stress–susceptible marginal environments (Murphy et al. 2014).

Take–all is one of the globally present diseases of barley (Kazemi et al. 2008), caused by the fungus *Gaeumannomyces graminis* var. *tritici* (Sacc.) v. Arx & Olivier var. *tritici* Walker (*Ggt*). Take–all manage–ment approaches in agricultural fields consist of crop rotation and tillage (Liu et al. 2009). Resistant cultivars of barley to take–all are not available and methods to control this disease by fungicides are insufficient. Fungicides are often dangerous to apply and cause serious environmental concerns. Therefore, investigation for other control methods such as biological control is necessary.

Colonization of plant roots by endophytic fungi may confer benefits to the host such as enhanced resistance to pathogens and improved stress tolerance or improved plant growth. Needless to say, endophytic fungi of barley can have high agricultural significance (Murphy et al. 2013). Studies have shown that *Microdochium bolleyi* (syn.: *Idriella bolleyi*) is a frequent and successful endophyte in plant roots, particularly those of grasses, such as wheat, barley, oats, native and invasive pasture grass and beach grasses (Sieber & Grunig 2013). *Microdochium bolleyi* form typical dark septate endophytic structures in the roots (David et al. 2016). *Microdochium bolleyi* exhibited suppression of different foliar and soilborne plant pathogens including *Septoria nodorum* (Sieber et al. 1988), *Fusarium culmorum* and *Bipolaris sorokiniana* (Duczek 1997, Knudsen et al. 1995) and *G. graminis* var. *tritici* (Kirk & Deacon 1987).

Microdochium bolleyi has been identified as a potential agent for the biocontrol of *Ggt* in wheat (Jadubansa et al. 1994). Although wheat is the main host for *Ggt*, barley, acts as a host (Monfort et al. 2005b), and barley is severely attacked by this pathogen in the west of Iran (Yosefvand et al. 2015).

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The main objectives of this study were the identification of *M. Bolleyi* as an endophytic fungus in barley roots, and determination of its ability in suppressing take–all disease. Improving barley growth under laboratory conditions was also investigated.

MATERIALS AND METHODS

During the spring of 2014, healthy barley plants were collected from fields in Kermanshah province, west of Iran. Twenty healthy plants were carefully uprooted and immediately transferred to the laboratory in plastic bags under cold conditions for further processing. The root samples were rinsed under running tap water to completely remove soil and debris. The roots were cut into five mm fragments and sterilized with 96% ethanol for 1 min, soaking for 3 mins in sodium hypochlorite (2% available chlorine v/v) and 96% ethanol for the 30s, and finally rinsing three times in sterile distilled water to remove surface sterilization agents (Larran et al., 2007). Nine pieces of root samples placed in potato dextrose agar (PDA) medium containing chloramphenicol (50 $mg.l^{-1}$) and dishes were incubated at 25 ºC for nine days.

Morphological identification of fungal isolates

In order to morphological identification of the fungal isolates, the colony color, shape and size of phialides, shape and size of spores and chlamydospores were examined by a light microscope as described by Hernandez–Restrepo et al. (2016). Photographs were taken using the BH2 Olympus microscope and thirty measurements of each type of structure were made using BioloMICSMeasure software.

Sequencing and phylogenetic analyses

In order to confirm the morphological identification, genomic DNA of two isolates (B26 and W2) was extracted using the methods described by Gardes et al. (1993). PCR amplification carried out by using primers ITS1 and ITS4 (White et al. 1990).

corresponding to the ITS region, in a final volume of 25 µl, by the following program: an initial denaturation step at 94 ºC for 3 min; then 30 cycles, consisting of denaturation (30 s at 94 ºC), annealing (30 s at 50 ºC), and extension (2 min at72 ºC); and a final extension step of 10 min was allowed at 72 ºC before cooling or removing the tubes. The amplified DNA was then sequenced in Macrogen Co. (South Korea) and compared with other fungal DNA sequences which deposited in GenBank (NCBI) database (www.ncbi. nlm.nih. gov/genbank/) using the BLAST search tool. Homologous fungal ITS regions were retrieved from NCBI and a phylogenetic tree was constructed using the neighbour–joining method in MEGA5 (Hall, 2013), with 1000 bootstrap replicates.

***In vitro* antagonistic bioassay**

Biocontrol assay was carried out by the dual–culture method. W2 and B26 were evaluated to prove their biocontrol effect against *Ggt* obtained from Mycology Collection of Kermanshah Agricultural Research Center. Petri dishes containing PDA were inoculated with one plug (5mm in diameter) of *Ggt*. Another plug containing *M. bolleyi* was placed at a distance of 3 cm from *Ggt* plug after 7 days. Petri dishes without *M. bolleyi* plug served as control. The percentages of inhibitions of radial growth of *Ggt* were measured two weeks after inoculation as describing by Royse & Ries (1978).

Growth tube experiment

Barley seeds were rinsed under tap water for 1 h and then surface sterilized by soaking in 5% sodium hypochlorite (NaOCl) for 1 h. Seeds were rinsed three times in sterile distilled water, then dry–blotted onto the sterilized filter paper *under sterilized conditions*, and pre–germinated on water agar for one day at 25 °C. As for growth–promoting effect, young seedlings were transplanted singly to 30 ml autoclaved vermiculite in plastic tubes and inoculated with four plugs (5 mm in diameter) of *M. bolleyi* grown on PDA. Control plants were inoculated with four plugs of PDA disc (Maciá–Vicente et al. 2008). After ten days, growth parameters such as roots and aerial length, the fresh and dry weight of plants also were measured. Fungal colonisation of root pieces was recorded, and developing fungal colonies were isolated on PDA for identification. Percentage of root colonisation by *M. bolleyi* was then calculated as N_d/N_t*100 (Eq.1), where N_d is the number of root pieces from which the fungi were detected and N_t the total number of root pieces (Maciá–Vicente et al. 2009). Regarding suppression of disease test, simultaneous inoculation of barley roots with both *M. bolleyi* and *Ggt*, was performed as described by Macia–Vicente *et al*. in 2008. Control plants were inoculated with two plugs of *Ggt* without *M. bolleyi*. All culture tubes were kept in a growth chamber with a photoperiod of 16/8 h light/dark cycle at 25 °C for ten days (Macia–Vicencte et al. 2008b). The biocontrol activity of this endophytic fungi was measured by evaluating the growth parameters and percentage of necrosis roots disease severity (pathogenicity index), which was scored from 0 to 5 as follows: 0 = roots and crowns without necrotic spots; 1 = root and crown does not have one or more symptoms of necrotic spots; 2 = root and crown necrotic spots going without symptoms; 3 = more than 50% root necrosis 4 = roots almost black with 75% nigrescence crown development; 5 = root and crown black and dried plant (Khanahmadi et al., 2016).

Statistical analysis

In general, the experiment was conducted twice in a completely randomized design with four replications. It was composed of the following

treatments: (*i*) control plants without any fungi, (*ii*) plants with *M. bolleyi*, (*iii*) plants with *M. bolleyi* and *Ggt*, and (*iv*) plants with *Ggt*. Significant differences ($P < 0.05$) among the mean values of different treatments were calculated and evaluated using Duncan's Multiple Range Test on a statistical analysis system (SAS Institute Inc., USA).

RESULTS

Morphological and cultural characteristics

In this study, two isolates of recovered fungi (W2 and B26) from healthy barley roots were determined as *M. bolleyi* based on morphological and cultural characteristics that was described by Hernandez–Restrepo et al. (2016). The isolates formed microconidia, sporodochia, and chlamydospores. Colony texture was sticky without any aerial mycelium in the centre or over the entire colony. The colony color varied from white to dark (Fig. 1 a, b). Two distinct types of conidiogenous cells formed: ampullate and cylindrical (Fig. 1 e), which the size of these types were 3.2–6.3 × 2.6–3.9 µm and 2–2.7 × 1–1.4 µm, respectively. Dark chlamydospores were formed singly, in chain or clusters and 5.5–14.6 × 4.5–11.6 µm in size (Fig. 1 c, d). The microconidia were non–septate, hyaline, smooth, cylindrical, and 6.4 × 1.9 µm in size (Fig. 1 f).

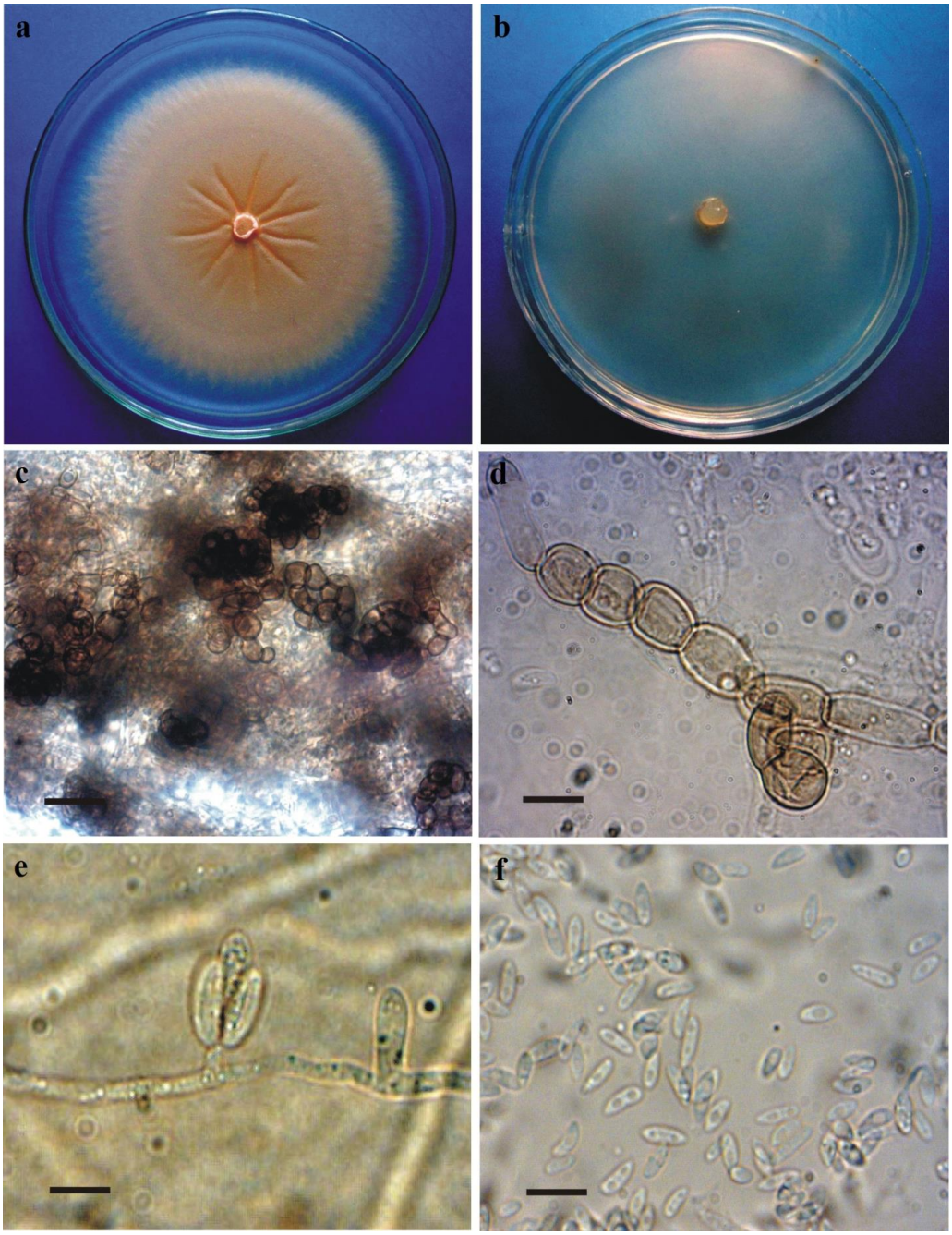

Fig. 1. *Microdochium bolleyi*. **a.** Colony on PDA after 14 days, **b.** Colony on MEA after ten days, **c–d.** Chlamydospores formation in chain and cluster on PDA medium, **e.** *conidiogenous cells*, **f.** Conidia. — Scale bars (c, f) = 10 µm, (d, e) = 5 µm.

Sequencing and phylogenetic analysis

An amplicon of about 500 bp was obtained for two isolates of the *Microdochium*. Sequencing analysis of two isolates (B26 and W2) (Accession Nos. KX343031 and KX343032) showed 100 % homology with valid sequences of *M. bolleyi* previously identified and deposited in GenBank (Accession No. HQ703412). Our isolates placed in the same clade with *M. bolleyi* from other authors with high bootstrap value in the ITS phylogenetic (Fig. 2). A culture of *M. bolleyi* (B26) was deposited in the Iranian Research Institute of Plant Protection (Iran 2726C).

Antagonism examination of *Microdochium bolleyi*

Both two isolates of *M. bolleyi*, that were evaluated in the dual culture tests, showed the antagonistic property against *Ggt*. Isolates B26 and W2 of *M. bolleyi* showed 20% and 45%, growth inhibition of *Ggt* respectively (Fig. 3 A, B). Isolate W2 was selected for barley colonization experiments, due to its percentage of growth inhibition of *Ggt* was over twice more than isolate B26.

***Microdochium bolleyi* effect on growth parameters**

In the laboratory test, colonization of barley roots by *M. bolleyi* had a clear plant growth–promoting effect on barley plants (Table 1). The results showed *M. bolleyi* dramatically raised all growth parameters ($P < 0.05$), root fresh weight (60.0%), aerial fresh weight (38.46%), root length (4.54%), aerial length (7.21%), root dry weight (60.43%) and aerial dry weight (38.60%). This endophytic fungi had the biggest effect on weight of barley roots, both dry and fresh roots, which was around twice as more as the figure for the barley roots without any fungi. Re–isolation of *M. bolleyi* in PDA media measured by Eq.1 and showed that the percentage of root colonization was 100% (Fig. 3 D, E).

***Microdochium bolleyi* effect on take–all disease**

In order to evaluate the effects of *M. bolleyi* on Take–all disease, after ten days, pathogenicity index, and also growth parameters were measured. Regarding pathogenicity index, the percentage of necrosis root disease severity was scored from 0 to 5 as was mentioned above. Statistical analysis demonstrated barley plants that were inoculated with *Ggt* and treated with *M. bolleyi* reduced remarkably index of root disease by 86.36%. As for growth parameters, *M. bolleyi* also significantly increased root fresh weight by 31.21%, aerial fresh weight by 15.15%, root length by 3.0%, aerial length by 2.35%, root dry weight by 30.94% and aerial dry weight by 12.28%. This meant that there was a significant variation of the growth parameters between the control plants inoculated with *Ggt* only and the barley plants inoculated with *Ggt* plus *M. bolleyi*, in 5% level (Table 1) (Fig. 3 C).

DISCUSSION

In recent decades, biocontrol strategy to reduce plant diseases has become of interest in integrated disease management (Soytong et al. 2001).

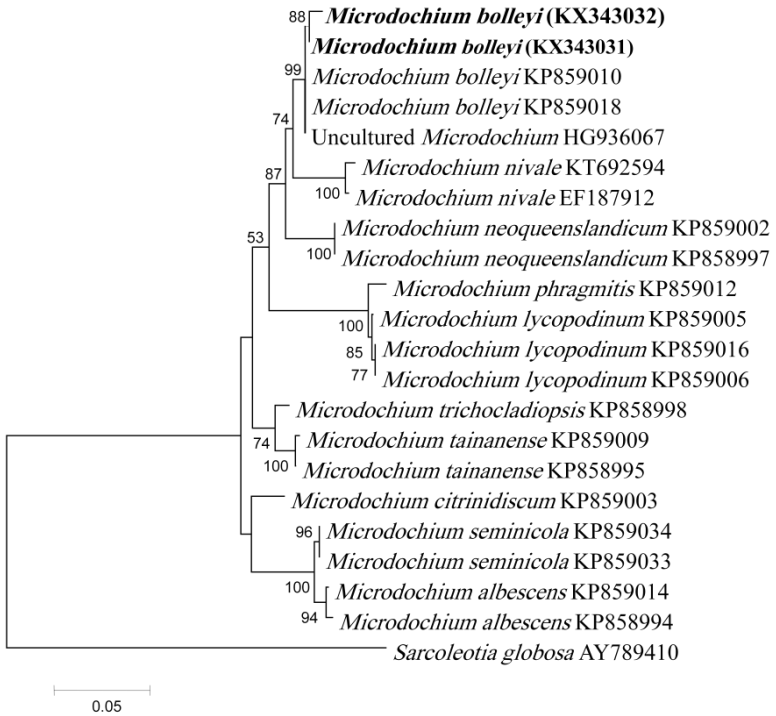

Fig. 2. The Phylogenetic tree was constructed by the neighbour–joining method based on entire ITS sequences of nuclear rDNA. Bootstrap values > 50% (1000 replicates) are shown next to the branches. *Sarcoleotia globosa* (AY789410) was used as outgroup taxon.

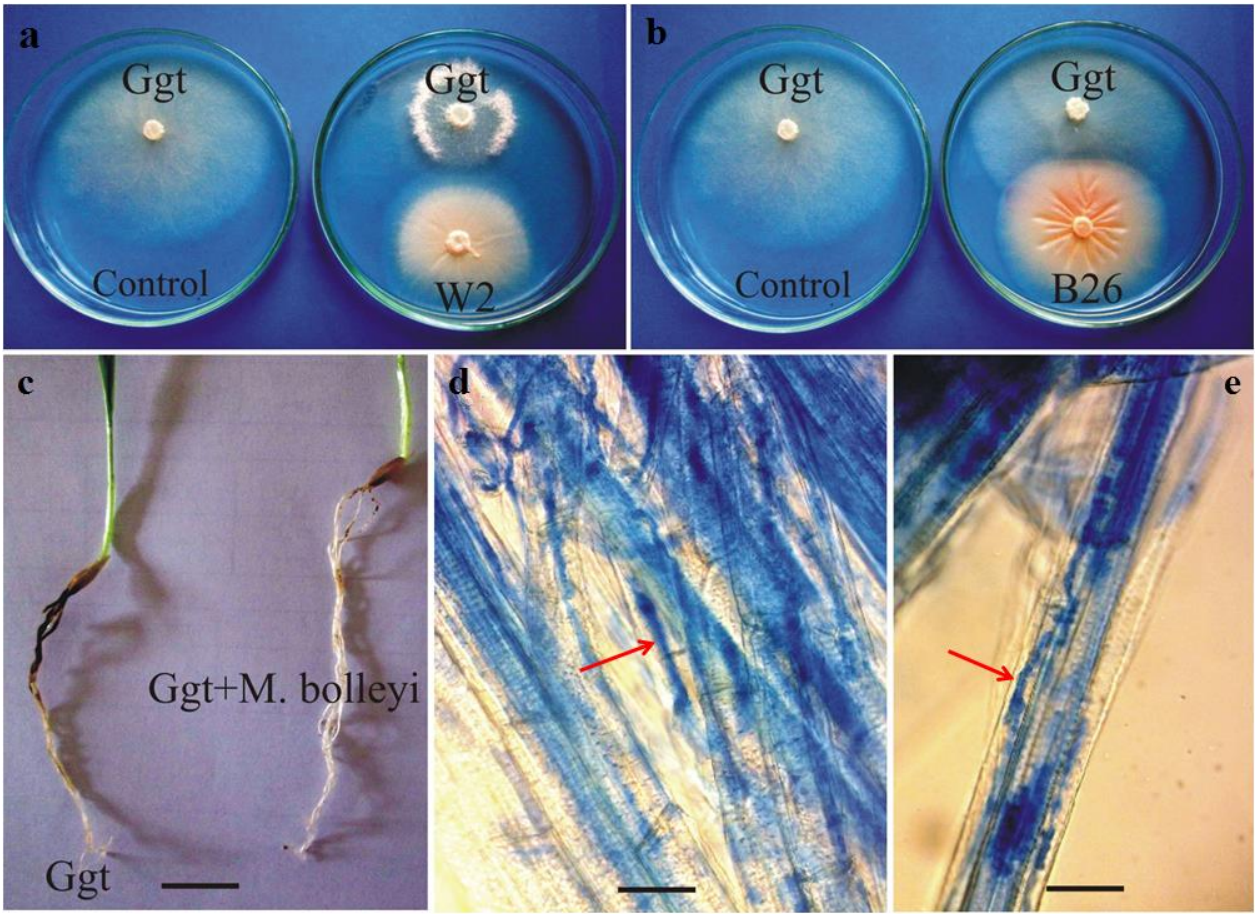

Fig. 3. a–b. Biocontrol activity of *Microdochium bolleyi* on *Gaeumannomyces graminis* var. *tritici* in the dual culture tests; c. black rot in root of barley inoculated with Ggt (left) and biocontrol activity of *M. bolleyi* on Ggt (right); d–e. Colonization of barley roots by *M. bolleyi* hyphae. — Scale bars (c) = 1 cm, (d, e) = 10 µm.

Table 1: Effect of inoculation with *Microdochium bolleyi*, *M. bolleyi* and *Gaeumannomyces graminis* var. *tritici* and *Ggt* alone on growth parameters of barley.

Treatments	Root fresh Weight (g)	Aerial fresh Weight (g)	Root Length (cm)	Aerial Length (cm)	Root dry Weight (g)	Aerial dry Weight (g)	Pathogenecity index
Control plants without any fungi	$0.0375^{c}\pm0.0025$	$0.0975^{b}\pm0.0025$	$13.7750^{b}\pm0.1887$	$14.2250^{b}\pm0.1031$	$0.0187^{c}\pm0.0012$	$0.0487^{b}\pm0.0012$	$0.00^{a}\pm1.00$
Plants with *M. bolleyi*	$0.0600^{a}\pm0.0041$	$0.1350^{a}\pm0.0050$	$14.4000^{a}\pm0.0408$	$15.2500^{a}\pm0.1041$	$0.0300^{a}\pm0.0020$	$0.0675^{a}\pm0.0025$	$0.00^{a}\pm1.00$
Plants with *M. bolleyi* and *Ggt*	$0.0475^{b}\pm0.0025$	$0.0950^{b}\pm0.0029$	$13.7500^{b}\pm0.0866$	$14.1250^{b}\pm0.0479$	$0.0237^{b}\pm0.0012$	$0.0457^{b}\pm0.0015$	$0.6^{b}\pm1.00$
Plants with *Ggt*	$0.0362^{c}\pm0.0024$	$0.0825^{c}\pm0.0025$	$13.3500^{c}\pm0.0288$	$13.8000^{c}\pm0.1080$	$0.0181^{c}\pm0.0012$	$0.0407^{c}\pm0.0005$	$4.4^{c}\pm1.00$

Values in the table are mean ± standard error (n=4). The different letter within each column indicates a significant difference among treatments ($P < 0.05$) using Duncan's Multiple Range Test

Fungal endophytes are ubiquitous colonizers of plant tissues where they do not normally cause any substantial morphological changes and disease symptoms. Many reports have been revealed that endophytic fungi are able to increase the growth of plants and they can suppress plant pathogenic fungi. In the research here, two fungal endophytic isolates (B26 and W2) obtained from healthy barley roots, has been identified as *M. bollyi*, using morphological criteria and ITS–rDNA gene analysis. The figures of plants growth–promotion test, demonstrated that inoculation of *M. bolleyi* (W2) on barley roots improved remarkably the growth of the host plant. Likewise, plant growth enhancements as a result of endophytic colonization, such as *Fusarium equiseti*, *Phoma* sp. and *Trichoderma virens* has been described previously (Saldajeno & Hyakumachi 2011). Re–isolation of *M. bolleyi* (W2) illustrated this

isolate colonized barley roots completely, at 100 percent, which was supported by the microscopic method (Figur 3. D, E).

Similarly, previous work reported a high percentage of root colonization of barley root by other endophytes such as *F. equiseti* and *Pochonia chlamydosporia* after seven days (Macia–Vicente et al. 2009). With respect to the take–all suppressing effect of *M. bolleyi*, inoculation of *M. bolleyi* on barley roots in growth tube showed it reduced pathogenicity index and improved growth parameter, which both were significant in 5% level, compared to *Ggt* alone. This result corroborates findings of previous studies where *M. bolleyi* controls take–all fungus. For instance, Kirk & Deacon (1987) and Lascaris & Deacon (1991) demonstrated *M. bolleyi* significantly reduced infection of wheat roots by *Ggt*. Moreover, several authors reported *M. bolleyi* has a potential for suppression other pathogens. For example, it had inhibition effect on *B. sorokiniana* and *F. culmorum* (Duczek 1997, Knudsen et al. 1995).

Previous research proposed the mechanisms that involved in suppressing of *Ggt* and other soil–borne root pathogens by an endophytic fungus. Liljeroth & Bryngelsson (2002) proposed that *M. Bolleyi* induced systemic resistance in barley because they found that it could reduce disease symptoms caused by *Bipolaris sorokiniana* in leaves where *M. Bolleyi* did not exist physically. Another possible mechanism is competence for space, in fact, Maciá–Vicente et al. (2008b) figured out it was the main mechanism of disease suppression in their research. Furthermore, evidence from Monfort et al. (2005) illustrated that the promotion of plant growth was the mechanism by which egg–parasitic nematophagous fungi reduce *Ggt* in barley roots. Kirk & Deacon (1987) proposed a competition for colonization of cortical cells are the mechanism of suppressing *Ggt* by *M. bolleyi* in cereal roots. In our experiment, *M. bolleyi* showed antagonism in dual cultures to *Ggt*. likewise, *M. bolleyi* are capable of producing antifungal compounds (Zhang et al. 2008), so these metabolites could also be a plausible reason for such a phenomenon. In addition, we guess competition for space and plant growth promotion was an important factor for reduction of disease symptoms caused by *Ggt*. To our knowledge, this study is the first report of *M. bolleyi* in Iran. Kirk & Deacon (1987) and Duczek, (1997) pointed to it has several unique properties that make it an appropriate candidate for commercial control of take–all. Hence, we propose that further studies are required for the application of this fungus as biological control agents in field conditions against soilborne pathogens in Iran.

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اثر مهار زیستی قارچ اندوفیت ریشه جو، *Microdochium bolleyi*، در برابر *Gaeumannomyces graminis* var. *tritici*

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چکیده: بیماری پاخوره غلات، ناشی از قارچ خاکزاد (*Ggt*) *Gaeumannomyces graminis* var. *tritici*، یکی از مهم‌ترین بیماری‌های خاکزاد گندم و جو در ایران و سایر کشورها است. در این مطالعه دو جدایه از جنس *Microdochium* (W2 و B26) از ریشه سالم گیاهان جو در مزارع استان کرمانشاه در سال ۱۳۹۳ جداسازی شد. پس از بررسی‌های ریخت‌شناختی و مولکولی، این جدایه‌ها به‌عنوان *Microdochium bolleyi* شناسایی شدند. مطالعه کنترل زیستی به روش کشت متقابل نشان داد که جدایه‌های W2 وB26 به‌ترتیب ۴۵٪ و ۲۰٪ رشد شعاعی *Ggt* را مهار کردند. جدایه W2 برای بررسی اثر مهار زیستی *Ggt* و همچنین توانایی بهبود رشد گیاه میزابان، به ریشه جو مایه‌زنی شد. به‌منظور بررسی اثر سرکوب بیماری، شاخص بیماریزایی (درصد شدت بیماری ریشه‌های نکروتیک) و پارامترهای رشدی اندازه‌گیری شد. شاخص بیماریزایی برای گیاه جو مایه‌زنی شده با *Ggt* به‌علاوه *M. bolleyi*، ۰/۶ و برای گیاه جو تیمار شده با *Ggt* به‌تنهایی، ۴/۴ بود. *M. bolleyi* همچنین طول ریشه را ۳ ٪، طول اندام هوایی را ۲/۳۵ ٪، وزن تر ریشه را ۳۱/۲۱ ٪، وزن تر اندام هوایی را ۱۵/۱۵ ٪، وزن خشک ریشه را ۳۰/۹۴ ٪ و وزن خشک اندام هوایی را ۱۲/۲۸ ٪ افزایش داد، که در سطح احتمال پنج درصد معنی‌دار بود. برای مطالعه اثر افزایش رشد میزبان، پارامترهای رشدی اندازه‌گیری شد. نتایج نشان داد *M. bolleyi* طول ریشه را ۴/۵۴ ٪، طول اندام هوایی را ۷/۲۱ ٪، وزن تر ریشه را ۶۰/۰ ٪، وزن تر اندام هوایی را ۳۸/۴۶ ٪، وزن خشک ریشه را ۶۰/۴۳ ٪ و وزن خشک اندام هوایی را ۳۸/۶۰ ٪ افزایش داد. تحلیل آماری نشان داد بین گیاه شاهد و گیاه تیمار شده با *M. bolleyi* در سطح احتمال پنج درصد اختلاف معنی‌دار وجود داشت. این اولین گزارش از قارچ *M. bolleyi* برای فلور قارچی ایران است و می‌تواند به عنوان یک عامل مهار زیستی در آینده مورد استفاده قرار گیرد.

واژه های کلیدی: مهارکننده زیستی، افزایش رشد گیاه، پاخوره غلات

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