INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–0368/201x/00–0–000–000
DOI: 10.17957/IJAB/15.0810
http://www.fspublishers.org

Full Length Article

Biocontrol Effect of a Novel Strain LB-1 on *Exserohilum turcicum* and its Growth-promoting Effect on Maize

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Abstract

LB-1 is a biocontrol strain newly screened in our laboratory. Previous work indicated that LB-1 exhibits strong and broad-spectrum activity against plant pathogenic fungi *in vitro*. In this study, the taxonomic assignment, the inhibition effect of LB-1 against *Exserohilum turcicum* and its growth-promoting effect on maize plant were investigated. Based on internal transcribed spacer (ITS) analysis, LB-1 was identified to be *Chaetomium subaffine*. LB-1 showed a good antagonistic effect on *E. turcicum* on dual-cultured PDA plate with an inhibition rate of 63.38%. The cell-free culture broth of LB-1 inhibited the colony extension of *E. turcicum* through poison plate assay with an inhibition rate as high as 73.59%. Furthermore, swollen and shrunken hyphae of *E. turcicum* at the colony contact surface were observed in dual culture assay, and the dissolved mycelia of *E. turcicum* were found in poison plate assay. The cell-free culture broth of LB-1 could also promote the seed germination and seedling growth of maize, but no plant growth-promoting effect was detected from LB-1 mycelia. All these results indicated that strain LB-1 exerted excellent biocontrol effect on *E. turcicum* and growth-promoting effect on maize plant.

Keywords: *Chaetomium subaffine*; Inhibitory effect; Growth-promoting; *Exserohilum turcicum*; Maize plant

Introduction

Northern corn leaf blight is the most common disease in maize (*Zea mays* L.) caused by *Exserohilum turcicum*, which causes long spindle-shaped necrotic lesions in maize leaves, sheaths and bracts, as well as changes in the growth and development of maize plants (Perkins and Pedersen, 1987; Dong, 2010). Presently, the field control of northern corn leaf blight mainly depends on use of chemicals and cultivation of resistant varieties (Sartori *et al*., 2017). Biocontrol agents reported for controlling northern corn leaf blight were *Trichoderma harzianum*, *Pseudomonas fluorescens*, *Aspergillus niger* (Harlapur *et al*., 2007), *Serratia plymuthica* (Wathaneeyawech *et al*., 2015) and *Bacillus subtilis* (Sartori *et al*., 2017). Recently, the biocontrol effects of *Chaetomium* spp. have been reported constantly and *C. globosum* was one of the most investigated (Soytong *et al*., 2001; Shanthiyaa *et al*., 2013; Phong *et al*., 2016). In addition, *C. spirale* (Guo *et al*., 2005), *C. cupreum* and *C. lucknowense* (Hung *et al*., 2015) on plant disease control were also discussed, but biocontrol of northern corn leaf blight with *Chaetomium* spp. was not reported.

Many biocontrol agents are endophytes in plants. These endophytes can benefit the growth and the disease resistance of plants through their own metabolic activities (Palaniappan *et al*., 2010; Dutta *et al*., 2014). Nejad and Johnson (2000) found that endophyte from oilseed rape not only has significant inhibitory effects on plant pathogenic fungi of *Verticillium dahliae* and *Fusarium oxysporum* f. sp *lycoperisci*, but also has growth-promoting effects on tomato and oilseed rape plants. Abdallah *et al*. (2016) screened two biocontrol strains of *Stenotrophomonas maltophilia* S37 and *Bacillus mojavensis* S40 from *Datura stramonium* and found that these two kinds of strains can inhibit the *Fusarium oxysporum* f. sp. *lycopersici* and promote the growth parameters of plant heights, root length and fresh weight of tomato. Liu *et al*. (2016) separated a variety of biocontrol strains from *Cephalotaxus hainanensis* and found that 17.7% of these endophytes were found to possess disease-suppressive and plant growth-promoting effects.

LB-1 is an endophytic fungus isolated from the leaves of *Sabina chinensis* cv. Kaizuka by our laboratory in 2013, and preliminary dual culture assay experiments revealed that LB-1 exhibited antifungal effects on several plant pathogenic fungi in which *E. turcicum* was the most inhibited. In this study, the taxonomic assignment, the inhibitory effect of LB-1 on *E. turcicum* both under *in vitro* and *in vivo* conditions and the plant growth-promoting effects of LB-1 on maize were investigated. The aim of this

study was to provide a reference for further exploration and application of LB-1 in biocontrol of northern corn leaf blight.

Materials and Methods

Fungal Isolates and Culture Medium

The biocontrol strain LB-1 and the phytopathogenic fungus *Exserohilum turcicum* were cultured on potato dextrose agar (PDA, contained 20% potato, 2% dextrose and 2% agar). Cell-free culture broth of LB-1 was obtained with liquid shake culture in potato dextrose broth (PDB, contain 20% potato and 2% dextrose).

Molecular Identification of LB-1 with rDNA-ITS Analysis

Mycelia were obtained by scraping 6-day-old colonies of LB-1 from PDA plates and ground to powder with the aid of liquid nitrogen. Genomic DNA was extracted with CTAB method (Porebski *et al*., 1997). The quality and integrity of the extracted DNA was detected by agarose gel electrophoresis and amplified by PCR with a final volume of 50 μL containing 25 μL 2×Es *Taq* Master Mix (CWBIO, China), 1 m*M* genomic DNA (2 μL), and 10 μM primer of each fungal universal primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (2 μL for each primer). PCR amplification was performed using a thermal cycler (Eppendorf, USA) with initial denaturation at 94℃ for 5 min; 35 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 2 min and a final extension at 72℃ for 10 min (Hermosa *et al*., 2000). The amplified DNA was sequenced in both directions with the initial PCR amplification primers by Bgi Gene Technology Company (China). The tested DNA sequence was compared with the ITS sequences of organisms deposited in GenBank of National Center for Biotechnology Information (NCBI). Phylogenetic tree was produced by neighbor-joining (NJ) method (Sugiyama and Mikawa, 2001).

Detection of the Antagonistic Effects of LB-1 on *E. turcicum*

Dual culture assay was used to determine the antagonistic effect of LB-1 on *E. turcicum* as described by Hung *et al*. (2015). Briefly, 6-mm-diameter mycelial discs of stain LB-1 and *E. turcicum* were obtained from the periphery of 3-day-old colonies. Two mycelial discs from each of LB-1 and *E. turcicum* were placed opposite on PDA plate and incubated for five days (25℃, natural light). PDA plate with single mycelial disc of *E. turcicum* served as control. Radii of *E. turcicum* on single-cultured PDA plate (A) and dual-cultured PDA plate toward LB-1 (B) were measured, the inhibition rate was calculated as follows and the antagonistic effect of LB-1 was evaluated. Inhibition rate (%) = (A – B)/A × 100. Five replicates were set for each treatment and control.

Inhibitory Effect of LB-1 Cell-free Culture Broth on *E. turcicum*

Cell-free culture broth of LB-1 was obtained according to the method described by Sha *et al*. (2012): ten mycelial discs of LB-1 were inoculated in Erlenmeyer flask containing 300 mL of PDB, shaking cultured in a water bath oscillator (THZ-320, Jinhong, Shanghai, China) at 25℃ and 130 rpm for 20 days. The culture broth was filtrated with three layers of sterile gauze, the filtrate was centrifuged at 4℃ and 12000 rpm for 15 min and the supernatant was used as cell-free culture broth of LB-1.

Poison plate assay was used to determine the inhibitory effect of LB-1 cell-free culture broth on *E. turcicum* as described by Benfradj *et al*. (2016). The cell-free culture broth of LB-1 was passed through a 0.22 μm millipore filter and mixed with autoclaved PDA culture medium at a proportion of 1:3 (v/v). A 6-mm-diameter mycelial disc of *E. turcicum* was placed on the center of the PDA plate and incubated for 6 days at 25℃ with natural light. PDA plate amended with sterile distilled water was used as blank control. Colony diameters of *E. turcicum* on PDA plates were measured, the inhibition rate was calculated and the effect of LB-1 cell-free culture broth on *E. turcicum* was analyzed. Inhibition rate (%) = (A – B)/A × 100, where A is the colony diameter of control and B is the colony diameter of treatment. Five replicates were set for each treatment and control.

Microscopic Observation of the Characteristics of *E. turcicum*

Mycelia of *E. turcicum* at the colony contact surface on dual-cultured PDA plate and on LB-1 cell-free culture broth containing PDA plate were obtained six days post inoculation (dpi). Wet-mount slides were made, and the characteristics of *E. turcicum* were observed and photographed with a phase-contrast inverted microscope (TS100, Nikon, Tokyo, Japan).

Analysis of the Biocontrol Effect of LB-1 on *E. turcicum* in Potted Maize Seedling

Seeds of the maize cultivar Shihai 928 (susceptible to *E. turcicum*) were sown in pots (30 cm in diameter, filled with sterile soil) and incubated in greenhouse free from other plant pathogens (17-25℃, natural light).

LB-1 suspension was obtained by dissolving pre-cultured LB-1 mycelia into sterile distilled water and diluted to approximately 1×10^5 cfu mL^{-1}. *E. turcicum* spore was got from pre-cultured colonies and dissolved in sterile distilled water to obtain spore suspension of *E. turcicum* with a concentration of approximately 1×10^5 spore mL^{-1}.

Maize seedlings at 5-7 leaf stages were inoculated with spore suspension of *E. turcicum* (25 mL per pot) by foliar spraying. LB-1 cell-free culture broth and LB-1 suspension were foliar applied individually in two ways: one was sprayed (25 mL per pot) on the leaves of maize seedlings 24 h before inoculation and the other was sprayed 1 and 3 dpi. The treated maize seedlings were incubated in greenhouse at 17-25℃ with natural light and insulated from other plant pathogens.

Tween 20 (0.03%) was supplemented in cell-free culture broth and suspension of LB-1 to facilitate their adhesion to maize leaves when being foliar applied (Fawe *et al*., 1998), corresponding concentration of Tween 20 was used as a treatment. Sterile distilled water was used as blank control. Ten pots (3 maize seedlings per pot) were used for each treatment and control. Disease index was calculated 10 dpi according to the Guidelines for the Field Efficacy Trials (II) - Part 107, National Standard of China. Control efficacy was calculated as: control efficacy (%) = (1 – disease index of treatment/disease index of control) × 100.

Detection of the Growth-promoting Effect of LB-1 on Maize

Growth-promoting effect of LB-1 on seed germination was analyzed as follows: maize seeds of Shihai 928 were sterilized with 5% NaClO for 3 min, washed with sterile distilled water for five times, placed on two layers of sterile filter paper in Petri dish, soaked with four different solution treatments individually and incubated at 25℃ and the moistness of the filter papers was maintained. Seed germination rate and radicle length were detected 7 days later as described by Cassán *et al*. (2009). The four treatment solutions were (1) LB-1 cell-free culture broth, (2) one day with LB-1 cell-free culture broth and the other 6 days with sterile distilled water, (3) LB-1 suspension, (4) one day with LB-1 suspension and the other 6 days with sterile distilled water. A total of 100 maize seeds were set for each treatment. Seed soaked with sterile distilled water was used as blank control.

Three application ways of LB-1 were used to investigate its effect on the growth of maize seedling as follows: (1) seed soaking: seeds of Shihai 928 were sterilized as previously described and soaked in suspension and cell-free culture broth of LB-1 for 24 h individually. The treated seeds were sown in pots and incubated in greenhouse (17-25℃, natural light); (2) root irrigation: pre-incubated maize seedlings with the same growth tendency were selected at 3-4 leaf stages and root irrigated with suspension and cell-free culture broth of LB-1 (100 mL per pot) individually and incubated continuously in greenhouse (17-25℃, natural light); (3) foliar spraying: maize seedlings with the same growth tendency were selected at 3-4 leaf stages, foliar sprayed with suspension and cell-free culture broth of LB-1 (25 mL per pot, with 0.03% Tween 20) individually and continuously incubated in greenhouse (17-25℃, natural light). The plant height, stem length, root length, diameter of stem and fresh weight of maize seedlings with different treatments were detected 30 days after sowing. The growth-promoting effects of LB-1 and its cell-free culture broth on maize seedlings were analyzed. 0.03% Tween 20 was set as the positive control. Maize seedling treated with sterile distilled water was used as blank control. Ten pots, each with 3 maize seedlings were set for each treatment and control.

Statistical Analysis

Data was expressed as mean ± standard deviation. The least significant difference (LSD) test was conducted using the SAS system (Version 8.1, SAS Institute, USA) with the threshold for statistical significance set at $P = 0.05$.

Results

The Characteristics of rDNA-ITS Sequence and the Taxonomic Assignment of LB-1

The molecular analysis on LB-1 found that the PCR product of rDNA-ITS of LB-1 was 527 bp, which showed 99% similarity with those *Chaetomium* spp. deposited in GenBank of NCBI. Phylogenetic analysis on the sequence of rDNA-ITS indicated that LB-1 was more related to *Chaetomium subaffine* than to other *Cheatomium* spp. (Fig. 1).

Antagonistic Effect of LB-1 on *E. turcicum in vitro*

Dual culture assay showed that the colony extension of *E. turcicum* on dual-cultured PDA plate was significantly suppressed by rapid growth of LB-1 (Fig. 2b), with an inhibition rate of 63.38%. Swollen and shrunken hyphae of *E. turcicum* were observed at the colony contact surface of dual-cultured PDA plates (Fig. 2c). This indicated that strain LB-1 has prominent antagonistic effect on *E. turcicum* and the effect could be obtained through living competition, which might lead to abnormality of the hyphae.

Inhibitory Effect of LB-1 Cell-free Culture Broth on *E. turcicum in vitro*

Poison plate assay showed that the colony extension of *E. turcicum* was inhibited on PDA plate amended with LB-1 cell-free culture broth, and an inhibition rate of 73.59% was obtained. As compared with the normal colony of *E. turcicum* in control PDA plate (Fig. 2a), colony of *E. turcicum* on LB-1 cell-free culture broth containing PDA plate was barren and sparse (Fig. 2d), and the mycelium was shrunken and dissolved (Fig. 2e). This result indicated that the cell-free culture broth of LB-1 was also effective in suppression of *E. turcicum*.

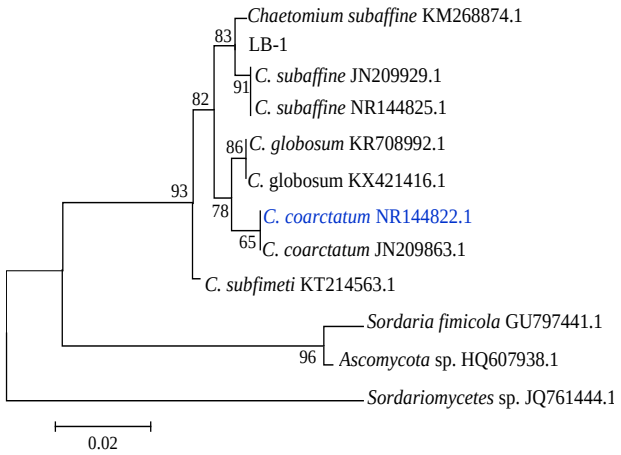

Fig. 1: The phylogenetic tree of strain LB-1 based on rDNA-ITS sequence

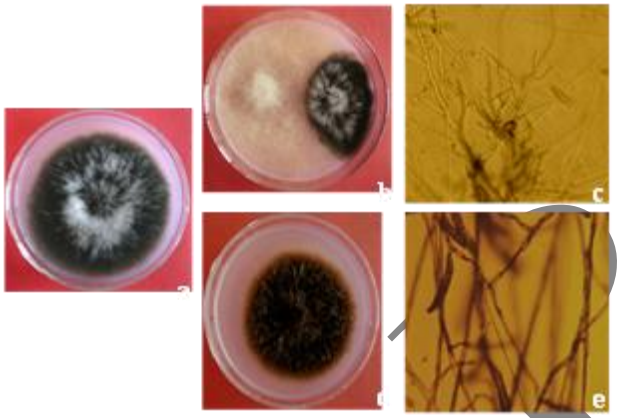

Fig. 2: Inhibitory effect of LB-1 on *E. turcicum*. a: Colony of *E. turcicum* on PDA plate; b, c: Colony and mycelia of *E. turcicum* on dual-cultured PDA plate with LB-1; d, e: Colony and mycelia of *E. turcicum* on PDA plate amended with LB-1 cell-free culture broth

Biocontrol Effect of LB-1 and its Cell-free Culture Broth on *E. turcicum* in Maize Seedling

The disease indices of northern corn leaf blight in potted maize seedlings were significantly reduced (P=0.05) by foliar spraying of LB-1 cell-free culture broth before or after the inoculation of *E. turcicum*, with control efficacies of 27.87% and 37.85%, respectively. The control efficacy detected from LB-1 suspension is only 5.27% and 7.91% before or after the inoculation of *E. turcicum*, respectively (Table 1). This indicated that LB-1 suspension has not obvious inhibitory effect on *E. turcicum* in maize seedling. 0.03% Tween 20 has the similar control efficacy on *E. turcicum* with that of the sterile distilled water, this excluded the interference effect of Tween 20 on the biocontrol evaluation of cell-free culture broth and suspension of LB-1 in the experiment.

Growth-Promoting Effect of LB-1 on Maize Seed Germination

Seed germination of maize cultivar Shihai 928 being treated with different treatments of LB-1 suspension and LB-1 cell-free culture broth are shown in Table 2, which indicated that there has no significant difference in seed germination among the treatments of LB-1 suspension, one day with LB-1 suspension and the other 6 days with sterile distilled water, one day with LB-1 cell-free culture broth and the other 6 days with sterile distilled water, and sterile distilled water (control), but the treatment of one day with LB-1 cell-free culture broth and the other 6 days with sterile distilled water was higher than other three treatments. Constant application with LB-1 cell-free culture broth can significantly (P=0.05) decrease the germination rate of the maize seed. There has no significant difference in the radicle length of maize seed treated with LB-1 suspension, one day with LB-1 suspension and the other 6 days with sterile distilled water, and sterile distilled water; the growth of radicle of maize seed was also suppressed by the treatment of constant application of LB-1 cell-free culture broth, whereas treatment of one day with cell-free culture broth and the other 6 days with sterile distilled water can significantly increase the radicle length of maize seed. These results indicated that proper application of LB-1 cell-free culture broth can prompt the germination and radicle elongation of maize seed.

Growth-Promoting Effect of LB-1 on Maize Seedling

The growth parameters of maize seedlings treated with different treatments of LB-1 suspension and LB-1 cell-free culture broth are shown in Table 3, which indicated that any treatment ways of LB-1 suspension has no effect on the growth of maize seedlings, and the same results were obtained from LB-1 cell-free culture broth applied through root irrigation and foliar spraying. However, the plant height, root length, stem length, fresh weight of maize seedlings were significantly increased by the treatment of soaking maize seed with cell-free culture broth of LB-1 for 24 h before sowing. Effects of 0.03% Tween 20 was similar with that sterile distilled water, it means that trace concentration of Tween 20 has no effects on the growth of maize seedling.

Discussion

Chaetomium spp. are widely distributed in natural environments (Soytong *et al.*, 2001). However, given the limited morphological and developmental characteristics, ITS analysis has been commonly used in the classification of this group of fungi (Asgari and Zare, 2011; Aggarwal *et al.*, 2013). ITS is the region between 18S rDNA and 28S rDNA in fungal rRNA genome, which is highly repetitive and variable among interspecies.

Table 1: Incidence of the northern corn leaf blight in potted maize seedlings treated with LB-1 suspension and LB-1 cell-free culture broth individually after and before the inoculation of *E. turcicum*

Treatments	Disease index	Control efficacy (%)
LB-1 cell-free culture broth before inoculation	0.56±0.08 b	27.87
LB-1 cell-free culture broth after inoculation	0.49±0.06 b	37.85
LB-1 suspension before inoculation	0.74±0.05 a	5.27
LB-1 suspension after inoculation	0.72±0.06 a	7.91
Tween 20	0.71±0.06 a	8.92
Distilled water	0.78±0.05 a	/

Mean ± standard deviation. Values with the same small letter in the same row mean non-significance (P>0.05)

Table 2: Rates of seed germination and radicle length of maize seed treated with different treatments of suspension and cell-free culture broth of LB-1

Treatments	Rate of seed germination (%)	Radicle length (mm)
Suspension	92.33±2.52 a	18.78±1.12 b
Cell-free culture broth	31.33±1.53 b	9.58±1.92 c
Suspension + distilled water	94.67±1.53 a	19.44±0.58 b
Cell-free culture broth + distilled water	99.33±0.58 a	25.64±2.14 a
Distilled water	98.00±1.00 a	19.09±1.63 b

Mean ± standard deviation. Values with the same small letter in the same row mean non-significance (P>0.05).

Table 3: Growth parameters of maize seedlings treated with different application ways of LB-1 suspension and LB-1 cell-free culture broth

Treatment		Plant height (cm)	Root length (cm)	Stem length (cm)	Diameter of stem (cm)	Fresh weight of plant (kg)
I	Seed soaking	25.92±3.22 a	13.55±1.93 a	5.66±2.22 a	0.75±3.43 a	0.33±1.66 a
	Root irrigation	27.13±3.99 a	13.17±2.76 a	5.98±2.02 a	0.79±3.01 a	0.38±3.34 a
	Foliar spraying	25.71±4.45 a	13.67±3.15 a	5.13±2.51 a	0.74±2.07 a	0.37±2.76 a
	Tween 20	27.21±2.45 a	14.32±2.15 a	5.55±2.13 a	0.76±2.13 a	0.37±1.66 a
	Distilled water	27.13±3.45 a	14.08±2.13 a	5.85±1.75 a	0.79±1.87 a	0.35±2.10 a
II	Seed soaking	36.33±3.18 a	18.99±3.01 a	7.08±3.45 a	0.81±2.53 a	0.66±3.12 a
	Root irrigation	26.15±3.66 b	15.12±2.66 b	5.08±1.09 b	0.74±2.26 a	0.41±2.66 b
	Foliar spraying	28.92±4.64 b	15.77±2.15 b	5.11±1.88 b	0.77±1.98 a	0.50±1.89 b
	Tween 20	27.21±2.45 b	14.32±2.15 b	5.55±2.13 b	0.76±2.13 a	0.37±1.66 c
	Distilled water	27.13±3.45 b	14.08±2.13 b	5.85±1.75 b	0.79±1.87 a	0.35±2.10 c

I indicates LB-1 suspension, II indicates LB-1 cell-free culture broth. Mean ± standard deviation. Values with the same small letter in the same row mean non-significance (P>0.05) among treatment of LB-1 suspension and LB-1 cell-free culture broth, respectively

Since mid-1980s, ITS region has been widely used in fungal systematic evolution and genetic relationships (Swann and Taylor, 1993; Sherriff *et al*., 1995; Iwen *et al*., 2002). In this study, we analyzed the phylogenetic tree of strain LB-1 based on its rDNA-ITS sequence and identified LB-1 as *Chaetomium subaffine.*

Dual culture assay, poison plate assay and inhibition zone assay are the common methods to detect and screen the biocontrol agents (Brunner *et al*., 2005; Siameto *et al*., 2010). Biocontrol effects of several *Chaetomium* spp. have been detected using these methods (Gao *et al*., 2005; Hung *et al*., 2015; Zhao *et al*., 2017), and researches also revealed that these *Chaetomium* spp. have obtained their biological effects through hyperparasitism, living competition and antibiotic substance production (Soytong *et al*., 2001; Zhang *et al*., 2013; Xu *et al*., 2014). In this study, flourishing growth of LB-1 and inhibited colony extension of *E. turcicum* were found on dual-cultured PDA plate, which indicated that living competition might be a technique for LB-1 to obtain its antagonistic effect. Inhibited growth of *E. turcicum* with scattered colonies and shrunken mycelia on PDA plates containing 25% cell-free culture broth of LB-1 were also observed. This indicated that some antibiotic substances, which can lead to shrinkage of fungal mycelia, were contained in the cell-free culture broth of LB-1. Thus, antifungal substance production might be another technique for LB-1 to exert its biocontrol effect and this needs to be investigated further.

Northern corn leaf blight caused by *E. turcicum* occurs worldwide. In this study, LB-1 cell-free culture broth exhibited significant biocontrol effects on *E. turcicum* in potted maize seedlings with foliar spray before or after inoculation, which might indicate that emphasis should be focused on the cell-free culture broth when LB-1 is being explored for field control of northern corn leaf blight in the future. However, no obvious inhibitory effect was obtained from LB-1 suspension, which illustrated that LB-1 can't act on *E. turcicum* directly and can't colonize in maize plant.

Endophyte is common microbial population in plants, which have beneficial effects on plants via growth promotion and disease-resistance induction (Palaniappan *et al*., 2010). Growth-promoting effect of endophyte can be

achieved by solubilization of bound iron and phosphorus to an easier absorption form for plants (Rodriguez and Fraga, 1999; Whipps, 2001; Taurian *et al*., 2010) and by producing regulators of plants (Ahmad *et al*., 2008). Our research found that the seed germination and seedling growth of maize can be promoted by seed soaking treatment with LB-1 cell-free culture broth, indicating that cell-free culture broth of LB-1 has plant growth-promoting potential; however, the reason of this promoting effect needs to be studied further.

Conclusion

Our research found that novel biocontrol strain *Chaetomium subaffine* LB-1 has antagonistic effect on the plant pathogenic fungus *E. turcicum* and resulted in abnormality of the pathogen. The cell-free culture broth of LB-1 has prominent inhibition effects on *E. turcicum* both under *in vitro* and *in vivo* conditions and could lead to shrunken and deformed hyphae of *E. turcicum*. Furthermore, well promoting effects on seed germination and seedling growth of maize were detected from proper application of LB-1 cell-free culture broth. These results provide a valuable reference for field utilization of LB-1 in biocontrol of northern corn leaf blight.

Acknowledgements

This work was supported by Natural Science Foundation of Shandong Province, China (ZR2014JL017).

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(Received 09 March 2018; Accepted 28 June 2018)