

Biocontrol potential of endophytic *Penicillium* spp. against strawberry anthracnose

Darya Zargar¹, Jahanshir amini^{2*}, Jafar abdollahzadeh²

1. Former MSc Student, Dept. of Plant Protection, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran

2. Associated Professor, Dept. of Plant Protection, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran

(Received: February 21, 2018 – Accepted: May 13, 2018)

ABSTRACT

Plant growth which promotes rhizobacteria (PGPR) has a potential role in controlling root-knot disease. To Strawberry anthracnose disease, caused by *Colletotrichum nymphaeae* is a major limiting factor in strawberry production in Kurdistan province of Iran. In this research, endophytic fungi were isolated from stem and crown organs of strawberry and their antifungal activity against *C. nymphaeae* was tested. The selected isolates were identified as *Penicillium hordei* and *Penicillium polonicum* based on morphology and molecular characteristics. Antagonistic effects of selected isolates were evaluated against *C. nymphaeae*, through dual culture, volatile and non-volatile compounds production mechanisms in *in vitro* and greenhouse conditions. In volatile compounds test, two isolates of *P. hordei* and *P. polonicum* were able to reduce *C. nymphaeae* growth (34.05 and 42.36%) compared with control. In addition, isolates of *P. hordei* and *P. polonicum* reduced the strawberry anthracnose disease severity significantly in greenhouse conditions (58.9 to 61.4%) compared to the control. According to the results both *in vitro* and greenhouse experiments, both isolates reduced the growth of pathogen. Therefore, the results of this research showed that it may be possible to manage strawberry anthracnose disease effectively by using *Penicillium* species as biocontrol fungus. To our best knowledge, this is the first research related to the effects of *Penicillium* species on strawberry anthracnose.

Key words: Biological control, *Colletotrichum nymphaeae*, endophytic fungi, strawberry.

INTRODUCTION

Strawberry anthracnose disease commonly caused by three species of the *Colletotrichum* viz. genus (*C. acutatum* sensu lato, *C. gloesporioides* sensu lato and *C. fragariae*) (Howard *et al.*, 1992; Freeman & Kata, 1997). It is the most important disease of strawberry in the world (Mass, 1998) and it is a major limiting factor in strawberry production in Iran and reached epidemic in the Kurdistan province in 2014 and 2015 (Karimi *et al.*, 2017). *Colletotrichum nymphaeae* was identified as the causal agent of strawberry anthracnose in Kurdistan province of Iran (Karimi *et al.*, 2017). Disease symptoms include sunken necrotic lesions that can occur on all aerial parts of the strawberry plant, including the fruit, leaf, flower, petiole, stolen, crown and root (Denoyes-Rothan *et al.*, 1999). *Colletotrichum nymphaeae* caused fruit rot and the

disease is characterized by large, sunken lesions that render the fruit unmarketable (Howard *et al.*, 1992; Karimi *et al.*, 2017). Strawberry anthracnose is controlled by chemical fungicides such as Benomyl, Thiabendazole and Azoxystrobin (Wedge *et al.*, 2007; LaMondia, 1995; Smith and Black, 1992), but frequent application these fungicides can have damaging effects on the environment health has led to the selection of resistant population of the fungus in the field. Therefore, it can occur fail in the control of pathogen (Chen *et al.*, 2014; Stadnik *et al.*, 2009). Considering the side effects of fungicides, an alternative method of controlling of plant disease is the application of fungal endophytes. They are microorganisms which colonize the internal plant tissues without causing any disease symptoms and compete with the pathogens for the same niches and thereby reduce disease

* Corresponding author E-mail: jamini@uok.ac.ir

severity in plant (Moreira *et al.*, 2014; Arnold, 2007). It plays a role as biocontrol agents against plant pathogens through competing for space, nutrition, antibiosis and induction of host defense (Chen *et al.*, 2014; Benhamou *et al.*, 1996; Strobet *et al.*, 1997). Strawberry anthracnose biocontrol by fungal endophytes may represent a potentially attractive and environmentally safe alternative since endophytes could better limit disease severity through inhibition of the systemic fungus progress. Also, endophytes may have other beneficial effects on plants, including enhanced growth, resistance to abiotic stresses and protection against plant pests (Rosenblueth & Martinez-Romero, 2006; Swarthout *et al.*, 2009; Vega *et al.*, 2008). In addition, to our knowledge there are very few studies about strawberry fungal endophytes and their effects on strawberry anthracnose. Therefore, these results have encouraged us to study the effectiveness of fungal endophytes in controlling the strawberry anthracnose disease. The aim of the present research was to evaluate their antifungal activity against *C. nymphaeae* causal agent strawberry anthracnose disease *in vitro* and greenhouse conditions.

Materials and methods

Pathogen and pathogenicity tests

The pathogenic agent was obtained from the collection of plant protection department of Tabriz University in Iran. The pathogen previously was identified using morphological characteristics and molecular sequence as *C. nymphaeae* (GenBank accession number CCTUCCh32) (Karimi *et al.*, 2017). It was grown on Potato Dextrose Agar (PDA) medium for 10 days at 25 °C and suspension were adjusted to 10⁶ conidia/ml by a hemocytometer.

Pathogenicity of selected pathogen at first was evaluated using the 50- day-old susceptible cultivar of strawberry (Paros) susceptible to *C. nymphaeae* under greenhouse conditions. The plants were sprayed until runoff with a suspension of *C. nymphaeae* (10⁶ conidia/ml) using a hand-held atomizer. Suspension conidial was prepared in sterile distilled water containing 0.1% (v/v) of Tween 20. Control plants were sprayed with sterile distilled water containing 0.1% (v/v) of Tween 20. All plants were incubated in plastic bags to maintain moisture for 72 h in the dark and then maintained at 25 ± 2 °C in greenhouse conditions. Disease severity was measured 60 days after inoculation of *C. nymphaeae* on petioles which mentioned another section (Delp & Milholland, 1980). Then, pathogenicity of pathogen was evaluated on strawberry fruits cv. Paros. Fruits were surface-sterilized by dipping in 70% ethanol for 30 s, rinsed three times in sterile distilled water, and fruits were dried on sterile paper under a laminar hood. A total of 15 µl of conidial water suspension (1 × 10⁶ conidia/ml) with 0.1 % of tween 20 (v/v) was pipetted onto the fruit surface (ten fruits were inoculated). Sterile distilled water plus 0.1 % Tween 20 was used as control. Inoculated fruits were incubated in plastic containers with moistened filter papers, at 22 °C, 90 % high relative humidity. After seven days, disease severity was measured using a scale of 0 to 5, where 0 represent healthy fruits and symptomless of disease, 1, 2, 3, 4 and 5 represent <20, 20.1 to 40, 40.1 to 60, 60.1 to 80, and 80.1 to 100% of the fruit area rotted respectively, which described by modified method of Huang *et al.* (2011) (Figure 2).

Isolation of endophytic fungi

Healthy strawberry plants (*Fragaria*

ananassa Duch Family Rosaceae) were collected in two growing seasons (April and May 2015 and 2016) in flowering and fruit stages from different strawberry-growing areas in fields of Kurdistan Province in west of Iran. Collected plants were washed in running water to remove soil particles on the roots and crown. Samples of crown and stem were cut into 2-cm-long segments. Then, pieces of samples were surface sterilized following the procedures described by Larran *et al.* (2007), briefly in 70% ethanol (v/v) for 1 min, 3 min in 5% sodium hypochlorite (v/v), and 30 s in 70% ethanol and rinsed with sterile water three times. Finally, samples blotted dry on sterilized filter paper, cultured on PDA supplemented by Doxycycline (150 mg/l) in petri dishes (9 cm in diameters) and incubated at 25 °C. After 7 days the fungal colonies were individually transferred to new PDA dishes and after growing, fungal cultures were purified by single spore. Pure cultured were stored at 4°C.

Screening of fungal endophytes for their efficiency against *C. nymphaeae*
Antifungal activity of the endophytic fungal isolates was assessed through dual culture, volatile and non-volatile organic compounds assay as follows:

Dual cultural

Antifungal activity of each isolates of endophytic fungi against *C. nymphaeae* was measured using the dual culture technique on PDA medium. Five-day-old mycelial disc plug (5mm in diameter) of *C. nymphaeae* was placed at one centimeter from the edge of plate and a mycelial discs plug (5 mm diameter) of fungal endophyte were placed opposite of the pathogen at equal distance. The plates were incubated at 25 ± 2 °C under a photoperiod of 12 h

for seven days. The percentage growth inhibition zone (I) against pathogen was measured using the following formula:

$I \text{ (Percentage)} = [(d_c - d_t)/d_c] \times 100$,
where d_c is radial growth of pathogen in control, d_t is radial growth of pathogen in dual plate culture (treatments).

Volatile compounds

Experiment was done using the two-sealed base-plates method described by Huang *et al.* (2011). A mycelial disc plug (5mm diameter) of fungal endophyte was placed on PDA medium in plates. In another plate containing PDA medium, a five mm disc of a 7 days old culture of *C. nymphaeae* was placed at the center of plate. Then, both half plates were placed face to face preventing any physical contact between the pathogen and endophytes isolates, so that plates of pathogen were placed inversely over a bottom dish containing endophytes. Plates were sealed with parafilm. In the control treatment, a bottom dish inoculated with *C. nymphaeae* was placed inversely over a bottom dish containing PDA without endophyte. Each treatment included four replicates. All plates were incubated at 25 °C for 10 days. The percentage of inhibition zone for each isolates was measured as per formula discussed previously.

Non-volatile compounds

A mycelial disc plug of each fungal endophyte was inoculated in 100 ml potato dextrose broth (PDB) in a 250 ml Erlenmeyer flask at 25 ± 2 °C in shake culture (120 rpm) for 10 days. Culture media was filtrated using 0.22 µm membrane filters and in order to remove hyphal fragments. Ten ml of filtrate culture were added to vial containing 90 ml PDA medium. Then, the medium was poured into plates (20 ml each), and after solidification each

plate was inoculated with one 5mm disc plug from the edge of a 10-day-old pathogen. The plates were incubated at 25 ± 2 °C under a photoperiod of 12 h. After seven days, the percentage of inhibition zone for each isolates was measured as per formula discussed previously.

Antagonistic activity of fungal endophytes against *C. nymphaeae* in greenhouse conditions

The suspension of endophytes and pathogen were vortexed and filtrated through two layer of cheesecloth. To determine the inhibition effect of fungal endophytes in greenhouse condition, conidial suspensions of each one of the fungal endophytes diluted with sterile distilled water (containing 0.1% of Tween 20, v/v) to a final concentration of 10^6 conidia/ml and sterile distilled water (as control) were applied to the strawberry plants (six week old) as a spray to run-off. The plants were individually covered with 0.1-mm-thick transparent plastic film to maintain moisture at 28 °C for 72 h in the dark. Then, conidial suspension of *C. nymphaeae* (10^6 conidia/ml) was sprayed on the inoculated strawberry plants until runoff and plants were incubated in greenhouse at 25 ± 2 °C, 60-70% relative humidity, 16 h light, 8 h darkness (Freeman *et al.*, 2001). Inoculated Plants with the pathogen until runoff using a hand-held atomizer were applied as control. After eight weeks, disease severity was determined on petioles using a scale of 0 to 5, where 0 represent healthy petiole without lesions; 1, 2, 3, 4, represent <3, 3-10, 10.1-20 and >20 mm petiole with lesions in length respectively; and 5 represent entirely necrotic petiole and plant is dead (Delp & Milholland, 1980). Biocontrol efficacy was measured using the following formulas

(Cao *et al.*, 2011):

$$\text{Biocontrol efficacy \%} = \frac{[(\text{disease index of control}) - (\text{disease index of treated})]}{(\text{disease index of control})} \times 100.$$

Identification of selected isolates of endophytic fungi

The identity of the selected endophytes was confirmed using a combination of phenotypic characteristics and molecular phylogenetic analyses. Phenotypic characteristics were made based on their colony characteristics, and microscopic observation of their hyphal, conidia and conidiophores morphology on Czapek yeast agar (CYA), malt extract agar (MEA) and yeast extract sucrose agar (YES) media at 25 ± 2 °C for 7 days. The morphological and microscopic observations were determined using Olympus BX51 microscope and DP71 digital camera (Houbraken *et al.*, 2016).

Phylogenetic characterization

The DNA of all endophytes was extracted by the protocol described by Reader and Broda (1985). Quality and concentration of extracted genomic DNA was utilized by spectrophotometer. PCR amplification of the calmodulin (CaM) gene was conducted using universal primers CMD5 (CCGAGTACAAGGARGCCTTC) and CMD6 (CCGATRGAGGTCATRAGGTGG) (Hong *et al.*, 2006). The PCR amplified sequences of endophytes isolates were searches for similarity to other sequences available in the NCBI database using basic local alignment search tool (BLAST) algorithm (<http://www.ncbi.nlm.nih.gov/>). Phylogenetic analyses were performed using neighbor-joining (NJ) methods using PAUP version 4.0b10 (Swofford,

2003). Statistical significance of the branching order was estimated by 1000 replication of bootstrap resampling of the original nucleotide sequence alignments.

Statistical analysis

Experiments *in vitro* and greenhouse conditions were designed as a completely randomized design (CRD). Data before analysis changed to normalcy by the formula $Arc \sin \sqrt{\%}$ ((Little & Hills, 1978). All analyses were performed using the SPSS (Statistical Product and Service Solutions) versions 16.0. All data are presented as mean \pm standard error of means. The means were compared by *Duncan's Multiple Range Test (DMRT)* at $P \leq 0.05$.

Results

Pathogenicity test

Pathogenicity of *C. nymphaeae* isolate

on strawberry plant cv. Paros was measured after 60 days. The first symptom of disease was appeared on petioles of inoculated strawberry plants c.v Paros as lesions and necrotic four weeks after inoculation. Mean of disease index for treatment (inoculated plants) after 60 days were observed at 4.0. Petioles of strawberry plants in control treatment were health had no symptoms (Figure 1).

Also, pathogenicity of *C. nymphaeae* isolate on strawberry fruits cv. Paros after seven days was calculated and mean of disease index was observed at 3.8 for inoculated fruits. Anthracnose symptoms was observed on inoculated strawberry fruits (except on the untreated control) such as black, sunken necrotic lesions and conidial ooze on fruits (Figure 2). Finally, the pathogen was re-isolated from plants exhibiting disease symptoms.

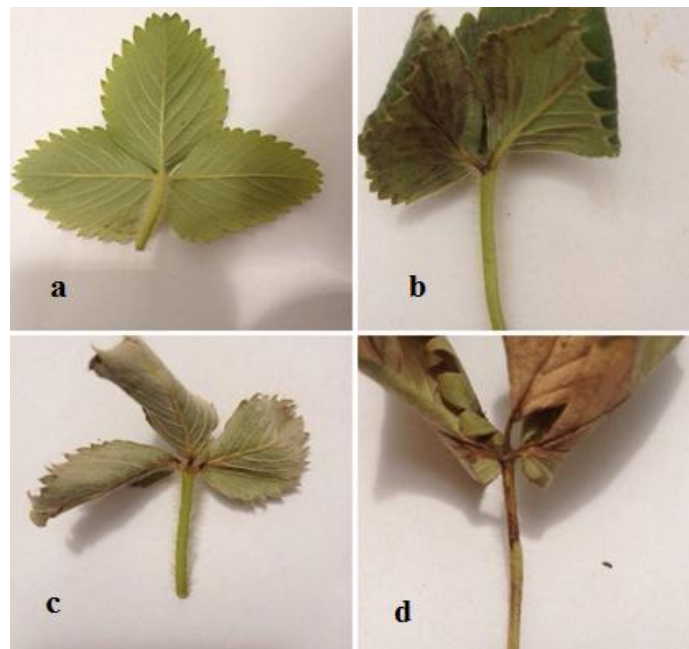


Figure 1. Symptoms of necrotic petiole after 56 days inoculation with *C. nymphaeae* based on 1-5 severity scale proposed by Delp and Milholland (1980). Control (a), inoculated treatment (b, c, and d). Mean of disease index of inoculated treatment = 4.0

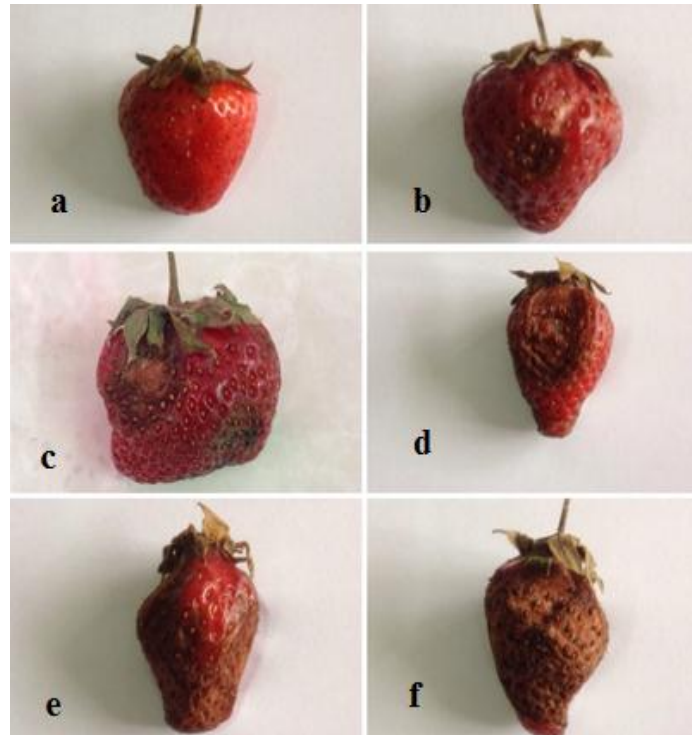


Figure 2. Symptoms of sunken necrotic lesion in each fruit was rated for disease severity using a scale of 0 to 5 was described by modified method of Huang et al (2011). Control (a, severity rate= 0), Treatments (b, c, d, e and f, where severity rate represent 1, 2, 3, 4, 5 respectively).

Mean of disease index of inoculated treatment = 3.8

Endophytic fungi isolation and their antagonistic activities

A total of 18 isolates of fungal endophytes were obtained from different pieces of strawberry plant such as stem (7 isolates), and crown (11 isolates). Antagonistic screening of these isolates was carried out *in vitro* on the mycelial growth of *C. nymphaeae*. Of the 18 isolates of endophytes, two isolates with more than 25% inhibition rate (in dual culture method) were selected for further tests. The selected isolates were obtained namely E25 and E40 which isolated from strawberry stem and crown respectively.

Identification of selected isolates

The selected fungal endophytes (E25 and E40) were subjected to morphological and molecular identification. Identified selected fungal endophytes were including: *Penicillium hordei*, *Penicillium polonicum*.

Colonies color of *P. polonicum* on CYA, MEA and YES after 7 days were blue green, black green and brown green respectively. Colonies reverse color on CYA, MEA and YES yellow, brown and yellow disposed to brown. Conidiophores of *P. polonicum* were terverticillate; metulae cylindrical with $10.2-11 \times 2.15-3.1 \mu\text{m}$; phialides were flask-shaped with $6.5-8.9 \times 2-2.4 \mu\text{m}$. Conidia were globose with smooth rough walled. Conidial dimensions were $2-3.2 \mu\text{m}$.

Colonies color of *P. hordei* on CYA, MEA and YES after 7 days were white disposed to green, black green, black green disposed to grey, but colonies reverse color on these media were white dispersed to yellow, yellow dispersed to brown and black yellow respectively. Conidiophores of *P. hordei* were terverticillate; metulae cylindrical with $8.6-11.3 \times 2.15-3.0 \mu\text{m}$; phialides were cylindrical with $6.5-$

8.8 × 2-3.3 µm. Conidia were globose with 1.9-3.0 µm.

Blast analysis of sequenced calmodulin gene homology and the phylogenetic analysis based on neighbor joining (NJ) method with 1000 bootstrap sampling revealed that E25 isolate belonged, with a similarity

of 100% to *P. hordei* CBS 701.68 and E40 isolate belonged, with a similarity of 100% to *Penicillium polonicum* CBS 222.28 (Figure 3). The Molecular identification of endophytic fungi isolates for the calmodulin sequencing genes listed in Table 1.

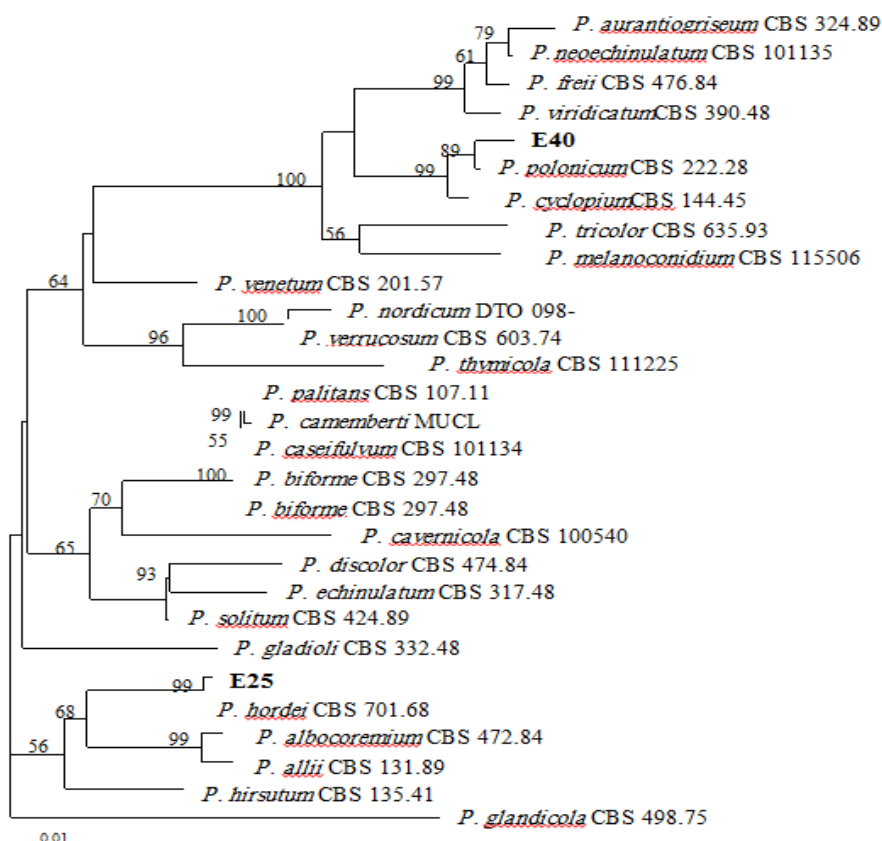


Figure 3. Phylogenetic tree of calmodulin genes sequence showing position of *Penicillium polonicum* and *Penicillium hordei* isolates (E40 and E25) with the sequences from selected references strains. Neighbor-joining trees resulting from calmodulin (CaM) gene sequences of the isolates derived from strawberry plant. Values of the branches are neighbor-joining bootstrap supports expressed as percentages. It is showing the relationship among members of *Penicillium* section Fasciculate (clade 1). The tree was rooted with *Penicillium glandicola* CBS 498.75

Table 1. Molecular identification of antagonistic fungal isolates using subunit calmodulin genes

Isolates	Blast research results	Identity%	Length (bp)	Homologue sequences
E25	<i>Penicillium hordei</i>	99	461	CBS 701.68
E40	<i>Penicillium polonicum</i>	99	458	CBS 222.28

Antagonistic activity of fungal endophytes against *C. nymphaeae* in vitro

Dual culture assay

Both two selected endophytes isolates

showed antifungal activities against the strawberry anthracnose *in vitro* in dual culture assay on PDA medium. Inhibition of growth ranged from 29.20 % to 31.80 % (Table 2).

Effects of volatile compounds on *C.*

nymphaeae growth

Two endophytic fungi isolates including *P. hordei*-E25 and *P. polonicum*-E40 reduced *C. nymphaeae* growth 34.05% and 42.36% respectively (Table 2).

Effects of non-volatile compounds on *C. nymphaeae* growth

Two selected endophytes isolates of *Penicillium* spp. showed the lowest effectiveness in inhibiting *C. nymphaeae* growth (7.83 % to 18.91 %) (Table 2).

Table 2. Growth of different endophytic fungi against *C. nymphaeae* on PDA *in vitro* after 7 days

Treatments	dual culture		Volatile compounds		Non- Volatile compounds	
	CD (cm) ^a	Inhib.% ^b	CD (cm) ^a	Inhib.% ^b	CD (cm) ^a	Inhib.% ^b
<i>P. hordei</i> -E25	2.66 ± 0.33 ^b	31.80	2.46 ± 0.11 ^b	34.05	3.41 ± 0.22 ^b	7.83
<i>P. polonicum</i> -E40	2.76 ± 0.33 ^b	29.20	2.15 ± 0.2 ^c	42.36	3.00 ± 0.11 ^b	18.91
Control	3.90 ± 0.33 ^a	-	3.73 ± 0.11 ^a	-	3.70 ± 0.15 ^a	-

Results are mean of four replicates with ± standard error (SE). Means followed by the same letter(s) within the column are not significantly different ($P \leq 0.05$) according to Duncan multiple-ranges test (DMRT). ^aCD: radial growth (cm) on potato dextrose agar; ^binhib: Percentage of mycelial growth inhibition.

Greenhouse experiment

Results of antagonistic effects of endophytes in greenhouse assay are shown in Table 3. The effect of endophytes on disease severity showed that great difference in disease reduction was observed between control

and treatments (ranged 58.9 to 61.4%). Both treatments significantly decreased disease severity and increased control efficacy compared to untreated control (pathogen alone) with over 58.9%. Infection index in control was 4 (Table 3).

Table 3. Effect of endophytic fungi against strawberry anthracnose disease (cv. Paros) in greenhouse conditions eight weeks after inoculation with *C. nymphaeae*

Treatments	Disease severity	Control efficacy (%)
<i>P. hordei</i> -E25	1.66 ± 0.28 ^b	58.9
<i>P. polonicum</i> -E40	1.56 ± 0.14 ^b	61.4
Non-treated	4.00 ± 0.39 ^a	-
Water control	0.00 ^c	-

Results in the column are mean of four replicates obtained in two experiments with ± standard error (SE). Means followed by the same letter(s) within the column are not significantly different ($P \leq 0.05$) according to Duncan multiple-ranges test (DMRT).

Discussion

Strawberry anthracnose is a destructive disease in the world (Mertely & Legard, 2004). Several *Colletotrichum* species including *C. acutatum*, *C. simmondsii*, *C. nymphaeae*, *C. fioriniae*, *C. salicis* and *C. godetiae* have been reported on strawberry worldwide (Karimi *et al.*, 2017; Damm *et al.*, 2012; Baroncelli *et al.*, 2015). Species of *C. nymphaeae* has become increasingly serious in Kurdistan Province in Iran. Soft and

juicy tissues of strawberry are easily attacked by this pathogen. It infects different parts of strawberry plant, including the root, crown, stem, leaf, petioles, flower and fruit (Peres *et al.*, 2005).

Control of strawberry anthracnose by traditional methods is ineffective due to the lack of resistance cultivars and the limited effectiveness of chemical fungicides (Chalfoun *et al.*, 2011). Therefore, using biocontrol agents

(BCAs) such as fungal endophytes to control strawberry anthracnose can be a more efficient and environmentally friendly alternative. In this research, two endophyte isolates were selected from a collection 48 fungi tested *in vitro* by dual culture method.

The results of this study indicated that the most of selected endophytes have antagonistic effects against *C. nymphaeae*, as revealed in the volatile compounds assay. It is observable that although non-volatile compounds showed some inhibitory effects on *C. nymphaeae* growth, but they are not considered the main and major mechanism of biocontrol in comparison with volatile compounds (Table 1).

Endophytic *Penicillium* spp (strains of *P. hordei* and *P. polonicum*) showed better antagonistic properties compared to control in habiting the growth of *C. nymphaeae*. *Trichoderma* species can to produce cell-wall degrading enzymes such as chitinase, β -1,3-glucanase and N-acetylglucosaminidases, which are responsible for the degradation of fungi mycelium (Ting & Chai, 2015; Qualhato *et al.*, 2013). *Penicillium oxalicum* reduced vascular wilts of tomato caused by *Verticillium dahlia* and *Fusarium oxysporum* f. sp. *lycopersici* under greenhouse and field conditions (Larena *et al.*, 2003; Sabuquillo *et al.*, 2006).

The mechanisms of action employed by the isolates of selected endophytic fungi to control *C. nymphaeae* were not a subject of our investigation, but antibiosis is probably involved, because these isolates produced volatile metabolites. Other mechanisms of action such as induction of resistance and competition for space and nutrients may be having occurred. Fungal endophytes can colonize the internal plant tissues without causing visible symptoms and plays a role as biological

control agents (BCAs) against fungal diseases (Petrini, 1991; Arnold *et al.*, 2003). Fungal endophytes can protect plants against plant pathogens by three different mechanisms: direct effects (interaction between endophytes and pathogens by production of antibiosis), indirect effects (induction of systemic resistance) and ecological effects (competition for space and nutrients) (Gao *et al.*, 2010).

For example, the isolates of *Bacillus* sp. inhibited more than 60% of the *C. nymphaeae* conidial germination (Moreira *et al.*, 2014). Larran *et al.* (2016) showed that the endophytes (*Bacillus* sp. and *Fusarium* sp.) to inhibit conidial germination of *Drechslera tritici-repentis* causal agent of wheat tan spot disease significantly. Inhibitory of conidial germination and mycelial growth of *Botrytis cinerea* causal agent of fruit rot of strawberry by volatiles of *Candida intermedia* was also reported (Huang *et al.*, 2011). Our research confirms the report of Francesco *et al.* (2015), who reported that the volatile metabolite of *Aureobasidium pullulans* significantly reduced of conidial germination and the disease severity of *C. nymphaeae*, *Botrytis cinerea*, *Penicillium expansum*, *Penicillium digitatum* and *Penicillium italicum*.

Results of greenhouse tests indicated that the endophytic isolates E25 and E40 of *P. hordei* and *P. polonicum* significantly reduced the damage caused by the *C. nymphaeae* and reduced disease severity 58.9 and 61.4% compared to non-treated control.

However, to our knowledge, *Penicillium* spp. has not been previously reported as endophytic fungal isolated from strawberry plant and their beneficial effects i.e. strawberry anthracnose suppression under *in vitro* and greenhouse

conditions.

In conclusion, the results of our research indicated that selected endophytic fungi may be applied in the field to manage strawberry anthracnose and contribute to reduce chemical fungicide use and maintain the stability and durability of the resistance in strawberry cultivars against strawberry anthracnose disease. Therefore further research is needed to confirm the findings. Isolates of *Penicillium* spp.

that reduced the disease severity of strawberry anthracnose should be studied in greater detail with the aim of increasing their positive effect on diseased plant in field conditions.

Acknowledgements

The research was supported by the grant from the Department of plant Protection, University of Kurdistan (No 42/43757).

REFERENCES

- Arnold AE** (2007) Understanding the diversity of foliar endophytic fungi: progress, Challenges, and frontiers. *Fungal Biology Rev* 21: 51-66.
- Arnold AE, Mejia LC, Kylo D, Rojas EI, Maynard Z, Robbins N, Herre EA** (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences of the United States of America* 100: 15649-15654.
- Baroncelli R, Zapparata A, Sarrocco S, Sukno SA, Lane CR, Thon MR, Vannacci G, Holub E, Sreenivasaprasad S** (2015) Molecular diversity of anthracnose pathogen populations associated with UK strawberry production suggests multiple introductions of three different *Colletotrichum* species. *PloS One* 10 (6): e0129140.
- Benhamou N, Kloepper JW, Quadt-Hallmann A, Tuzun S** (1996) Induction of defense-related ultrastructural modification in pea root tissues inoculated with endophytic bacteria. *Physiology Plant Pathology* 112: 919-929.
- Cao Y, Zhang Z, Ling N, Yuan Y, Zheng X, Shen B, Shen Q** (2011) *Bacillus subtilis* SQR 9 can control Fusarium wilt in cucumber by colonizing plant roots. *Biology and Fertility of Soils* 47 (5): 495–506.
- Chalfoun NR, Castagnaro AP, Diaz Ricci JC** (2011) Induced resistance activated by culture filtrate derived from an avirulent pathogen as a mechanism of biological control of anthracnose in strawberry. *Biological Control* 58: 319-329.
- Chen Y, Gao X, Chen Y, Qin H, Huang L, Han Q** (2014) Inhibitory efficacy of endophytic *Bacillus subtilis* EDR4 against *Sclerotinia sclerotiorum* on rapeseed. *Biological Control* 78: 67-76.
- Damm U, Cannon PF, Woudenberg JHC, Crous PW** (2012) The *Colletotrichum acutatum* species complex. *Studies in Mycology* 73: 37–114.
- Delp B, Milholland R** (1980) Evaluation strawberry plants for resistance to *Colletotrichum fragariae*. *Plant disease* 64 (12): 1071-1073.
- Denoyes-Rothan B, Lafargue M, Guerin G, Clerjeau M** (1999) Fruit resistance to *Colletotrichum acutatum* in strawberries. *Plant Disease* 83: 549-553.
- Francesco AD, Uoglini L, Lazzeri L, Mari M** (2015) Production of volatile organic compounds by *Aureobasidium pullulans* as a potential mechanism of action against postharvest fruit pathogens. *Biological Control* 81: 8-14.
- Freeman S, Katan T** (1997) Identification of *Colletotrichum* species responsible for anthracnose and root necrosis of strawberry in Israel. *Phytopathology* 87: 516–

521.

- Gao FK, Dai CC, Liu XZ** (2010) Mechanisms of fungi endophytes in plant protection against pathogens. *African Journal Microbiology Research* 4: 1346-1351.
- Houbraken J, Wang L, Lee HB, Frisvad JC** (2016) New section in *Penicillium* containing novel species producing patulin, Pyripyropens or other bioactive compounds. *Personia* 36: 299-314.
- Howard CM, Mass JL, Chandler CK, Albregts EE** (1992) Anthracnose of strawberry caused by the *Colletotrichum* complex in Florida. *Plant Disease* 76: 976-981.
- Huang R, Li GQ, Zhang J, Yang I, Che HJ, Jiang DH, Huang HC** (2011) Control of postharvest Botrytis fruit rot of strawberry by volatile organic compound of *Candida intermedia*. *Phytopathology* 101: 859-869.
- Karimi K, Babai Ahari A, Arzanlou M, Amini J, Pertot I, Rota-Stabelli O** (2017) Application of the consolidated species concept to identify the causal agent of strawberry anthracnose in Iran and initial molecular dating of the *Colletotrichum acutatum* species complex. *European Journal of Plant Pathology* 147(2): 375-387.
- LaMondia JA** (1995) Inhibition with benomyl to growth *in vitro* of *Colletotrichum acutatum* and *C. fragaria* and Strawberry fruit infection by benomyl-resistant isolates of *C. acutatum*. *Advance strawberry Research* 14: 25-30.
- Larena I, Sabuquillo P, Melgarejo P, De Cal A** (2003) Biocontrol of Fusarium and Verticillium wilt of tomato by *Penicillium oxalicum* under greenhouse and field conditions. *Journal of Phytopathology* 151: 507-512.
- Larran S, Perello A, Simon MR** (2007) The endophytic fungi from wheat (*Triticum aestivum* L.). *World Journal of Microbiology and Biotechnology* 4: 565-572.
- Larran S, Simon MR, Moreno MV, Santamarina Siurana MP, Perello A** (2016) Endophytes from wheat as biocontrol agents against tan spot disease. *Biological Control* 92: 17-23.
- Little ETM, Hills FG** (1978) *Agricultural experimentation: Design and analysis*. John Wiley and Sons. Inc. New York. USA. 349 pp.
- Mass JL, (Ed.)** (1998) *Compendium of strawberry diseases*. 2nd ed. APS Press, St. Paul, MN.
- Mertely JC, Legard DE** (2004) Detection, isolation, and pathogenicity of *Colletotrichum* spp. from strawberry petioles. *Plant Disease* 88: 407-416.
- Moreira RR, Nesi VN, May De Mio LL** (2014) Bacillus spp. and *Pseudomonas putida* as inhibitors of the *Colletotrichum acutatum* group and potential to control Glomerella leaf spot. *Biological Control* 72: 30-37.
- Peres NA, Timmer LW, Adaskaveg JE, Correll JC** (2005) Lifestyle of *Colletotrichum acutatum*. *Plant Disease* 89: 784-796.
- Petrini O** (1991) *Fungal endophytes of tree leaves*. In: *Microbial Ecology of leaves*. Springer-Verlag, New York, 497 pp.
- Qualhato TF, Lopes FAC, Steindorff AC, Brandao RS, Jesuino RSA, Ulhoa CJ** (2013) Mycoparasitism studies of *Trichoderma* species against three phytopathogenic fungi: evaluation of antagonism and hydrolytic enzyme production. *Biotechnology Letter* 35: 1461-1468.
- Raeder U, Broda P** (1985) Rapid preparation of DNA from filamentous Fungi. *Letters in Applied Microbiology* 1: 17-20.
- Rosenblueth M, Martinez-Romero E** (2006) Bacterial endophytes and their interaction with hosts. *Molecular Plant Microbe Interaction* 19: 827-837.

- Sabuquillo P, De Cal A, Melgarejo P** (2006) Biocontrol of tomato wilt by *Penicillium oxalicum* formulation in different crop conditions. *Biological Control* 37: 256-265.
- Smith BJ, Black LL** (1992) *In vitro* activity of fungicides against *Colletotrichum fragariae*. *Acta Horticulturae* 348: 509-512.
- .Stadnik MJ, Araujo L, Valdebenito-Sanhueza RM** (2009) *Estrategias alternativas de controle da mancha foliar de glomerella e da saran da macieira*. In: Stadnik. M.J. (Ed.). *Manejo integrado de Doencas da Macieira*. CCA-UFSC, Florianopolis. P. 105-126.
- Strobet GA, Torzynski R, Bollon A** (1997) *Acremonium* sp. a leucinostain, a producing endophyte of European yew (*Taxus bacata*). *Plant Science* 128: 97-106.
- Swarthout D, Harper E, Judd S, Bultman T** (2009) Measures of leaf-level water-use efficiency in drought stressed endophytes infected and non-infected tall fescue grasses. *Environmental and Experimental Botany* 66: 88-93.
- Swofford DL** (2003) *Phylogenetic analysis using parsimony (and other methods)*. Version 4 Sunderland, Massachusetts Sinauer Associates.
- Ting ASY, Chai JY** (2015) Chitinase and β -1, 3-glucanase activities of *Trichoderma harzianum* in response towards pathogenic and non-pathogenic isolates: early indications of compatibility in consortium. *Biocatalysis and agricultural biotechnology* 4: 109-113.
- Vega FE, Posada F, Aime MC, Pava-Ripoll M, Infante F, Rehner A** (2008) Entomopathogenic fungal endophytes. *Biological Control* 46: 72-82.
- Wedge DE, Smith BJ, Quebedeaux JP, Constantin RJ** (2007) Fungicide management strategies for control of strawberry fruit rot diseases in Louisiana and Mississippi. *Crop Protection* 26: 1449-1458.