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Investigation of the antifungal activity of lichen (*Usnea longissima*) extracts against *Fusarium graminearum*

Asmar TAGHİYEVA[1], Ayşe Feyza TUFAN DÜLGER[2]*, Emre YÖRÜK[3], Tubanur ASLAN ENGİN[4]

[1]*Haliç University, Graduate Institute of Education, Molecular Biology and Genetics Program, İstanbul, Türkiye*
[2]*Ondokuz Mayıs University, Agriculture, Department of Agricultural Biotechnology, Samsun, Türkiye*
[3]*Istanbul Yeni Yüzyıl University, Faculty of Faculty Sciences and Literature, Department of Molecular Biology and Genetics, Istanbul, Türkiye*
[4]*Yalova University, Health Sciences Faculty, Department of Physiotherapy and Rehabilitation, Yalova, Türkiye*
**feyzadulger@omu.edu.tr, [1]esmer.tva @gmail.com, [3]emre.yoruk@yeniyuzyil.edu.tr, [4]tubanur.engin@yalova.edu.tr*

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Liken (*Usnea longissima*) ekstraktlarının *Fusarium graminearum*'a karşı antifungal aktivitesinin araştırılması

Abstract: The aim of this study was to investigate the antifungal effects and molecular changes caused by *Usnea longissima* Ach. extracts against *Fusarium greaminearum*. In agar well diffusion assay, the zone of inhibition increased as the concentration increased in both of methanol and acetone extracts (1, 10, 20 and 50 mg/ml). In terms of bioactivities, 1 mg/ml was active, while other concentrations were very active. At the molecular level, changes caused by 50 mg/ml methanol extract was analyzed by qPCR with terms of *cat*, *mst20*, and *tri5* genes, which are associated with antioxidation, apoptosis, and trichothecene production, respectively. Transcript levels of *tri5* decreased (0.29 fold) while *cat* (2.41 fold) and *mst20* (1.48 fold) increased. Findings from this study showed that *U. longissima* extracts could be natural antifungal agent against worldwide phytopathogen *F. graminearum*.

Key words: Agar well diffusion assay, *Fusarium graminearum*, gene expression, *Usnea longissima*

Özet: Bu çalışmanın amacı, *Usnea longissima* ekstraktlarının *Fusarium greaminearum*'a karşı neden olduğu antifungal etkileri ve moleküler değişiklikleri araştırmaktır. Agar kuyu difüzyon testinde, hem metanol hem de aseton özütlerinde (1, 10, 20 ve 50 mg/ml) konsantrasyon arttıkça inhibisyon bölgesi arttı. Biyoaktiviteler açısından bakıldığında, 1 mg/ml konsatrasyon aktifken, diğer konsantrasyonlar çok aktifti. Moleküler düzeyde, 50 mg/ml metanol özütünün neden olduğu değişiklikler, sırasıyla antioksidasyon, apoptoz ve trikotesen üretimi ile ilişkili *cat*, *mst20* ve *tri5* genleri açısından qPCR ile analiz edildi. *tri5*'in transkript seviyeleri azalırken (0.29 kat), *cat* (2.41 kat) ve *mst20* (1.48 kat) arttı. Bu çalışmadan elde edilen bulgular, *U. longissima* ekstraktlarının dünya çapında fitopatojen *F. graminearum*'a karşı doğal antifungal ajan olabileceğini göstermektedir.

Anahtar Kelimeler: Agar kuyu difüzyon testi, *Fusarium graminearum*, gen ekspresyonu, *Usnea longissima*

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1. Introduction

Fusarium graminearum Schwabe is the major causal agent of the Fusarium head blight of small grain cereals worldwide. The disease cause yield loss and decrease in product quality (Parry et al., 1995). In addition, contamination with mycotoxins such as deoxynivalenol, nivalenol and zearalenone during disease cause economic and health problems (Snijders, 1990; Desjardin, 2007; Osborne and Stein, 2007). Fungicides are generally used to prevent diseases. However, fungicides may cause environmental pollution as well as pathogenic fungi may develop resistance to fungicides. In the prevention of diseases, biological agents derived from different organisms can be considered as an alternative method by reducing the chemical input to the environment and being economical (Yuen and Schoneweis, 2007; Mielniczuk and Skwaryło-Bednarz, 2020). There are several examples of biological agents used in agriculture with their pesticide and insecticide activities (Yee and Toscano, 1998; Koch, 1999; Warrior et al.,1999; Lacey et al., 2011).

Lichens are an ecosystem complex that arise from a symbiotic association of an algae or cyanobacteria with fungi (Hawksworth and Grube, 2020). They are used for different purposes such as folk medicine, air pollution monitoring, dyeing clothes, cosmetics, and perfumery (Upreti et al., 2005; Joulain and Tabacchi, 2009; Aslan et al., 2013; Sharma and Mohammad, 2020). Lichens produce diverse range of secondary metabolites associated with various biological activities such as antibiotic, antibacterial, antifungal, antiviral, antiproliferative and cytotoxic effects (Shukla et al., 2010; Brisdelli et al., 2013; Furmanek et al., 2022). Although they have various activities, the agrochemical potential of lichens is not fully known.

Usnea longissima Ach. is a fruticose lichen produces unique secondary metabolites and has medicinal applications for centuries in Indian tradition (Halıcı et al., 2015; Reddy et al., 2019). There are limited studies on the antifungal effects of *U. longissima* extracts (Goel et al., 2011; Devashree et al., 2019; Yadav et al., 2021). However, there is no study on the effect of the *U. longissima* against world wide phytopathogen *F. graminearum*. The objective

of this study was to examine the in vitro antifungal effects and molecular changes caused by extracts of *U. longissima* against *F. graminearum*. In this context, methanol and acetone extracts were tested at concentrations of 1, 10, 20 and 50 mg/ml against *F. graminearum*. After seven days, the effects of treatments on fungal growth were determined by agar well diffusion assay and transcriptomic changes in *cat*, *mst20*, and *tri5* genes were investigated via qPCR.

2. Materials and Method

2.1. Preparation of *U. longissima* extracts

Two different organic solvent used for preparation of methanol and acetone extracts. Finely ground of dried *U. longissima* (10 g) were extracted in the relevant solvent (100 ml) according to Tiwari et al. (2011a). The dry extracts were stored at -20 °C. The extracts were dissolved in 5% dimethyl sulphoxide (DMSO) for further experiments.

2.2. Culture conditions of *F. graminearum*

F. graminearum PH-1 was grown in ½ strength potato dextrose agar (PDA) media at 25 ºC for 7 days. Then, 8-10 fungal discs were placed in carboxymethyl cellulose (CMC) media for macroconidia formation and incubated for 7 days at 28 °C, 100 rpm (Nalam et al., 2016). Concentrations of macroconidia were measured using a hemacytometer and standardized to $1x10^6$ macroconidia/mL.

2.3. Agar well diffusion assay

50 µl of a macroconidial suspension was spread on PDA medium. 5 mm of wells were punched from agar plates. Concentrations of 1, 10, 20 and 50 mg/ml extracts (60 µl) were loaded into the wells. Solvents were used as negative controls. The plates were kept at 25°C for 7 days. The zones of inhibition were measured using a ruler. Bioactivities of extracts were assessed as follows: very active, > 19 mm zone of inhibition; active, 13-19 mm zone of inhibition; partially active, 10-12 mm zone of inhibition; and inactive, < 10 mm zone of inhibition (Quinto and Santos, 2005). Experiments were performed with 3 technical and 3 biological replicates.

2.4. Total RNA isolation, cDNA synthesis and real time polymerase chain reaction (qPCR)

Total RNAs were extracted from *F. graminearum* PH-1 treated with methanol extract at a concentration of 50 mg/ml according to the manufacturer's protocol (NucleoSpin RNA Mini Kit, Macherey Nagel). The quantity and quality of the total RNAs were determined by a spectrophotometer and agarose gel electrophoresis.

Synthesis of cDNAs were performed from 1 µg of total RNA according to the manufacturer's protocol (ProtoScript® First Strand cDNA Synthesis BioLABs). Synthesized cDNAs were diluted to 20 ng/µl for further experiment.

Changes in the transcript levels of *cat*, *mst20*, and *tri5* genes were measured using qPCR. The reaction consisted of a total volume of 10 µL containing 5 µL of 1X Sybr Green I mix, 0.4 µM of each primers (Gazdağlı et al., 2018) and 2 µL of cDNA. Conditions were 2 min at 95°C, followed by 40 cycles of 5 s at 95 °C, 10 s at 58 °C, and 10 s at 72 °C. At the end of cycling, melting curve analysis was performed between 65 ºC - 95 ºC by increasing 0.5 ºC at 2-5 sec/step (Bio-Rad, CFX Connect RealTime System*). β-tubulin* was used as the internal control. Fold changes in gene expressions were determined by $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Experiments were performed in 3 technical and 3 biological replicates.

2.5. Statistical analysis

Gene expression analyses were performed using the one-way ANOVA and Tukey post-test using GraphPad Prism (Version 5.01) (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3. Results

3.1. Effects of extracts against *F. graminearum*

Antifungal activities of methanol and acetone extracts against PH-1 isolate were evaluated by in vitro agar well diffusion assay. On the 7th day of the assay, inhibition zones were observed in the extracts while the PH-1 control group covered the entire petri dish (Fig. 1). The zone of inhibition (mm) increased with the increase in the extract concentrations. In terms of bioactivity of methanol extract, 1 mg/ml of extract (14.5 mm) were active while 10, 20 and 50 mg/ml of extracts were very active (22.5 mm, 27.5 mm and 30 mm, respectively). Similarly, the acetone extracts were found active at 1 mg/ml (16.5 mm) while very active at 10, 20 and 50 mg/ml of concentrations (21 mm, 22.5 mm and 26.5 mm, respectively) (Table 1).

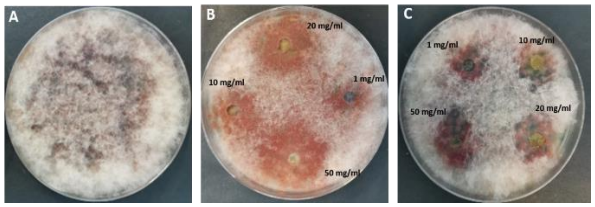

Figure 1. Agar well diffusion assay. (A) *F. graminearum* PH-1 control, (B) methanol and (C) acetone extracts of *U. longissima* at 1, 10, 20 and 50 mg/ml concentrations against PH-1.

Table 1. Zone of inhibition (mm) measurements in determining the bioactivities of methanol and acetone extracts of *U. longissima* against PH-1 through agar well diffusion assay. Error bars represent ± standard errors (SE) of three replicates.

Extract	Concentration	Inhibition zone (mm)
Methanol	1 mg/ml	14.5±0.7
	10 mg/ml	22.5±3.5
	20 mg/ml	27.5±3.5
	50 mg/ml	30±0
Acetone	1 mg/ml	16.5±2.1
	10 mg/ml	21.0±1.4
	20 mg/ml	22.5±3.5
	50 mg/ml	6.5±2.1

3.2. Gene expression analysis

To determine the molecular effect of 50 mg/ml methanol extract on PH-1 transcript levels of *cat*, *mst20*, and *tri5* genes were examined. A significant decrease was observed in the *tri5* gene, which is associated with trichothecene biosynthesis (0.29 fold, **). Significant increases were detected in the antioxidant-related *cat* gene (2.41 fold, ***) and the apoptosis-related *mst20* gene (1.48 fold, *) (Fig. 2).

4. Discussions

In this study, we firstly tested the *in vitro* antifungal effects of 1, 10, 20 and 50 mg/ml of methanol and acetone extracts of *U. longissima* against phytopathogenic *F. graminearum*.

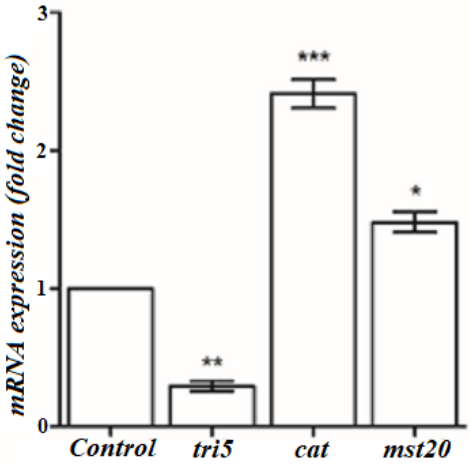

Figure 2. Fold changes of *tri5*, *cat* and *mst20* genes in PH-1 treated with 50 mg/ml methanol extract. Error bars represent ± standard errors (SE) of three replicates.

There are some reports testing the effect of lichen extracts on *Fusarium* Link. species. In a study by Tiwari et al. (2011a), it was determined that acetone and methanol extracts of *Bulbothrix setschwanensis* (Zahlbr.) Hale, *Everniastrum nepalense* (Taylor) Hale ex Sipman, *Heterodermia diademata* (Taylor) D.D. Awasthi, *Parmelaria thomsonii* (Stirt.) D.D. Awasthi lichens were effective against *F. oxysporum* Schltdl., *F. solani* (Mart.) Sacc. and *F. roseum* Link. In a study in which the antifungal activities of acetone, methanol and chloroform extracts of *Parmotrema tinctorum* (Despr. ex Nyl.) Hale were tested on ten plant pathogenic fungi, it was shown that the methanol extract was the most effective of all studied plant pathogens (Tiwari et al., 2011b). On the other hand, there are also some studies of lichen extracts in which inhibitory effects against *Fusarium* species were not observed. In a study with acetone extracts of Ramalina, no inhibitory activity was observed against *F. oxysporum*, *F. solani* and *F. verticillioides* (Sacc.) Nirenberg (Gazo et al., 2019). Similarly, acetone extracts of *Evernia prunastri* (L.) Ach., *Hypogymnia physodes* (L.) Nyl. and *Cladonia portentosa* (Dufour) Coem. lichen species were found to have no effect against *F. solani* (Halama and Van, 2004). There are limited studies on the antifungal, antimalarial, anti-insect, antimicrobial, antiquorum sensing, antioxidant, anti-urease, anti-elastase and antitumor effects of *U. longissima* lichen extracts (Goel et al., 2011; Yıldırım et al., 2012; Aydin et al., 2018, Dandapat and Paul, 2019; Devashree et al., 2019; Pamenta et al., 2020; Yadav et al., 2021; Bharti et al., 2022). In a study by Goel et al (2011), hexane, dichloromethane, ethyl acetate, methanol and aqueous extracts of four lichen species including *U. longissima* were tested on 9 pathogenic fungi, including *F. oxysporum*, and especially hexane and dichloromethane extracts were found to be the most active. However, there was no study on the effect of the lichen extracts against world wide phytopathogen *F. graminearum*. To our knowledge, we firstly reported that *U. longissima* extracts could exhibit a strong inhibitor activity against *F. graminearum*.

At the molecular level, we analyzed the alterations caused by the methanol extract in terms of antioxidant machinery (*cat*), apoptosis (*mst20*) and trichotocene production (*tri5*) in *F. graminearum*. We found that methanol extract induced antioxidant mechanism and apoptosis while suppressing trichothecene biosynthesis. Similarly, there are some reports of increased *cat* and *mst20* levels and decreased *tri5* level in determining the antifungal activity of the compounds (Gazdağlı et al., 2018; Shao et al., 2021; Teker et al., 2021; Xiu et al., 2021; Yörük et al., 2022).

Findings from this study showed that *U. longissima* extracts could be natural antifungal agent against worldwide phytopathogen *F. graminearum*. Considering the limited studies in the literature based on the antifungal effects of lichens, this study is unique and important in terms of contributing to research on biocontrol agent. To our knowledge, this is the first report that *U. longissima* extract inhibits fungal growth and DON biosynthesis in *F. graminearum*.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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