Effects of timothy Cladosporium eyespot on photosynthesis and biomass

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Abstract
Timothy is a forage mainly grown in Min County, Gansu Province, China. In 2021, a leaf spot disease outbreak on timothy grass occurred in Min County, adversely affecting its growth and productivity. Therefore, this study investigated the leaf spot disease incidence in Min County, morphologically and molecularly characterized the disease-causing pathogen, and assessed its effects on the growth, photosynthesis, and biomass of timothy seedlings re-inoculated with the isolated pathogen. In the field, the disease incidence on plants and leaves was 100 and 85%, respectively. Morphologically, young lesions were ellipsoidal–fusiform with dark purple margins and an off-white center, while the mature lesions were eye-shaped spots with a light brown center and dark purple edges. Molecular characterization identified the pathogen as Cladosporium phlei causing Cladosporium eyespot disease. The net photosynthetic rate, transpiration rate, fresh shoot weight, and dry shoot weight of timothy seedlings 14 days after inoculation with the pathogen were decreased by 29.77, 56, 45.45, and 46.42%, respectively, implying that Cladosporium eyespot disease is an important timothy grass disease in Min County. Therefore, developing an integrated control strategy is urgent to lessen the economic loss.

Keywords  Timothy1 · Forage plant2 · Hay yield3 · Fungal disease4 · grassland5

Introduction
Timothy (Phleum pratense L.) is a highly palatable grass which produces tender hay. It is considered the best for raising military and racing horses (Friedemann et al. 1999) as it adjusts their digestive function, reduces acute abdominal pain, and improves their endurance (Ragnarsson and Lindberg 2008). It is also used as pet food for dogs, cats, rabbits, and rodents (Martineau et al. 1994). Timothy is distributed in the cold temperate zone in the northern hemisphere and mountains in North America extending to Chile (Zhu et al. 2006). Its hay has a market price of $750–900 per ton (Cao 2003); thus, timothy grass is one of the main sources of income for farmers (Qi 2012). In China, timothy was first introduced in Min County from the USA in 1941 to raise military horses. It quickly became a promising industry given the cool and wet climate in Min County suitable for timothy growth (Hoglind et al. 2001). In addition, a new variety, Minshan timothy was bred by the Feed Technology Promotion Station of Gansu Province and the Military Horse Farm in Min County. It was registered by the China National Forage Variety Approval Committee in 1990 (Shi et al. 2020).

Up to 15 disease strains infecting timothy grass have been reported in China, including stripe rust (Puccinia striiformis) and Cladosporium eyespot (C. phlei) in eastern Gansu (Nan 1990), leaf spot (Hadorotrichum phragmicticum) and leaf blight (Brachysporium phragmitis) in Hebei, and net spot (Helminthosporium dictyoides) in Jilin (Jiang 1959).

C. phlei infects timothy grass, causing Cladosporium eyespot disease (Teruhiko et al. 1975). The disease is mainly distributed in Japan, South Korea, China, New Zealand, Austria, the United States of America, and Canada (Bensch et al., 2012). C. phlei: colonies maintained on PDA media for 2 weeks grow to 67 mm in diameter. The colonies have
pale olivaceous-grey to olivaceous-black, reverse fuscous-black, dark brick towards margins, releasing vinaceous pigment into the agar, with broad margins. The colonies are also feathery, regular, vinaceous-grey, floccose, and loose to dense, with aerial mycelium covering large parts of the colony and growth effusing with a somewhat elevated colony centre without prominent exudates (Bensch et al. 2012).

In 2021, a severe leaf disease affected the timothy grass in Min County, affecting its yields, nutrition, and aesthetic value and consequently reducing the farmers’ income. The leaf disease had characteristics similar to those of C. phlei, with the effects on photosynthesis and biomass of timothy remaining unclear. Therefore, the objective of this study was to identify the pathogen causing the leaf disease and its effects on photosynthesis and biomass of timothy. This study clarified the effects of the disease on the production of Timothy grass, and created a theoretical basis for the prevention and control of the disease.

Materials and methods

Field survey and sampling

An extensive survey was conducted in the primary commercial timothy producing regions of China from July to September 2021. The majority of the survey sites were in Min County, where the leaf disease was first reported. Min County is located in Gansu Province (103°41′–104°59′23″E, 34°07′34″–34°45′45″N) in the intersection zone of Qinghai–Tibet, loess and Mongolia plateaus. It has a plateau continental climate with annual average sunshine hours, temperature, relative humidity, frost-free days, and precipitation of 2214.9, 4.9 °C–7.0 °C, 68%, 90–120 days, and 596.5 mm, respectively. Its hottest and coldest months are July, and January, with an average temperature of 16 °C and −6.9 °C, respectively (Table 1).

In this study, three villages in Min County were identified, and five sites per village were mapped using Global Positioning System (Table 2). Next, five fields ranging from 1000 to 2500 m² per site were arbitrarily selected for Positioning System (Table 2). Next, five fields ranging from 1000 to 2500 m² per site were arbitrarily selected for Positioning System (Table 2). To determine the infection and control of the disease.

The total genomic DNA was extracted from pure colonies using the Ezup Fungal DNA Kit (Sangon Biotech), following the manufacturer’s protocol. The extracted genomic DNA was amplified in a 2720 thermal cycler (Applied Biosystems) in a total volume of 25 ml using ACT primers; 512F (5′-ATG TGC AAG GCC GGT TTC GC-3′)/783R (5′-TAC GAGTCCTTCTGGCCCAT-3′) and ITS1 (5′-TCCGTAGGG AACCTCAGGG-3′)/ITS4(5′-TTCCTCGGTTATGATAT GC-3′) as described by Bensch et al. (Bensch et al. 2012). The PCR products were visualized on 1.0% agarose gel, and

Table 1 Investigation areas of timothy Cladosporium eyespot (Gansu Province)

| City     | County | Township/Town | Specific address | East longitude | North latitude | Incidence rate | Number of diseased leaves collected | Time    |
|----------|--------|----------------|------------------|----------------|---------------|----------------|-------------------------------------|---------|
| Dingxi Min | Sigou  | Lilin          |                  | 104°01′66″      | 34°29′71″     | 100%           | 42                                  | 2021.09 |
| Dingxi Min | Sigou  | Benzhiyi       |                  | 104°02′77″      | 34°28′99″     | 100%           | 17                                  | 2021.09 |
| Dingxi Min | Lvjing | Hagu           |                  | 104°54′74″      | 34°32′51″     | 100%           | 22                                  | 2021.09 |
the positive products were purified and sequenced at Sangon Biotech. Sequence data were assembled using the DNAman software (version 5.2.2; Lynon Biosoft), and the sequences were deposited in the GenBank. Next, the phylogenetic tree was constructed using the Neighbor-joining (NJ) algorithm in the MEGA5.0 software.

**Pathogenicity tests**

Timothy grass seeds obtained from the farmers in Min County were sown in pots (height 15 cm, caliber 10 cm) in October 2021. Each pot contained 1.5 kg of double-autoclaved soil at 121 °C for 1 h with a 3-day interval between sterilizations. Seven seeds were sown per pot and maintained in a greenhouse at 25/20 °C day/night temperatures and 70% average relative humidity. After 3 weeks, *C. phlei* conidial suspension (1 × 10^6 conidia ml^-1) was sprayed on the seedlings, with seedlings sprayed with sterile water serving as controls. The severity of foliar symptoms and the disease incidence were evaluated on the seedlings 14-day post-inoculation. Next, the seedlings were uprooted, and their lengths and fresh weights determined. Finally, the leaf of each symptomatic

### Table 2 Strains of *Cladosporium* spp. Isolated from wild plants and crop species with collection details and GenBank accession numbers

| Species                  | Accession number | Host             | Country     | Collector     | GenBank accession numbers* | Reference       |
|--------------------------|------------------|------------------|-------------|---------------|----------------------------|-----------------|
| *Cladosporium acalyphae* | CBS 125,982      | *Acalypha australis* | South Korea | H.D.Shin     | HM147994 HM148481          | (Bensch et al. 2010) |
| *Cladosporium allicinum* | CBS 121,624      | *Hordeum vulgare* | Belgium     | J.Z.Groenewal | EF679350 EF679502          | (Schubert et al. 2007) |
| *Cladosporium angustisporum* | CBS 125,983 | *Alloxylon wickhamii* | Australia  | B.A.Summerell  | HM147995 HM148482          | (Bensch et al. 2010) |
| *Cladosporium asperulatum* | CBS 126,340     | *Protea susannae* | Portugal    | —             | HM147998 HM148485          | (Bensch et al. 2010) |
| *Cladosporium australiens* | CBS 125,984     | *Eucalyptus moluccana* | Australia | B.A.Summerell  | HM147999 HM148486          | (Bensch et al. 2010) |
| *Cladosporium basiiinflatum* | CBS 822.84    | *Hordeum vulgare* | Germany     | —             | HM148000 HM148487          | (Bensch et al. 2010) |
| *Cladosporium chabatense* | CBS 124,457     | *Pinus ponderosa* | Argentina   | A.Greslebin   | FJ936158 FJ936165          | (Schubert et al. 2009) |
| *Cladosporium colocasiae* | CBS 386.64      | *Colocasia esculentia* | Taiwan    | K.Sawada     | HM148067 HM148555          | (Bensch et al. 2010) |
| *Cladosporium delicatulm* | CBS 126,344     | *Tilia cordata*  | Germany     | K.Schubert    | HM148081 HM148570          | (Bensch et al. 2010) |
| *Cladosporium exile*     | CBS 125,987     | *Phyllactinia guttata* | USA       | D.Glawe      | HM148091 HM148580          | (Bensch et al. 2010) |
| *Cladosporium funicalosum* | CBS 122,129    | *Vigna umbellata* | Japan       | —             | HM148094 HM148583          | (Bensch et al. 2010) |
| *Cladosporium gansianum* | CBS 125,989     | *Streptizia sp.*  | South Africa | W.Gams      | HM148095 HM148584          | (Bensch et al. 2010) |
| *Cladosporium herbarum*  | CBS 121,621     | *Hordeum vulgare* | Netherlands | —             | EF679363 EF679516          | (Schubert et al. 2007) |
| *Cladosporium inversicolor* | CBS 401.80    | *Triticum aestivum* | Netherlands | —             | HM148101 HM148590          | (Bensch et al. 2010) |
| *Cladosporium perangustum* | CBS 125,996   | *Cassonia sp.*   | South Africa | P.W.Crous    | HM148121 HM148610          | (Bensch et al. 2010) |
| *Cladosporium phlei*     | CBS 358.69      | *Phleum pretense* | USA         | C.T.Gregory   | JN906981 JN907000          | (Bensch et al. 2012) |
| *Cladosporium phlei*     | LYZ0587         | *Phleum pretense* | China       | Yanzhong Li  | OM692481 OM721304         | Present study  |
| *Cladosporium phlei*     | LYZ0588         | *Phleum pretense* | China       | Yanzhong Li  | OM692482 OM721305         | Present study  |
| *Cladosporium phlei*     | LYZ0589         | *Phleum pretense* | China       | Yanzhong Li  | OM692483 OM721306         | Present study  |
| *Cladosporium phlei*     | LYZ0590         | *Phleum pretense* | China       | Yanzhong Li  | OM692484 OM721307         | Present study  |
| *Cladosporium phlei*     | LYZ0591         | *Phleum pretense* | China       | Yanzhong Li  | OM692485 OM721308         | Present study  |
| *Cladosporium phlei*     | LYZ0592         | *Phleum pretense* | China       | Yanzhong Li  | OM692486 OM721309         | Present study  |

*ITS internal transcribed spacer regions with 5.8S rRNA gene, ACT partial actin gene
and symptomless inoculated plant was used to reisolate the pathogen on PDA.

**Effect of the leaf disease on photosynthesis and plant growth**

Plants in six treatments and control pots were covered with black plastic bags for 48 h, then randomly arranged in an inoculation chamber. The effects of the leaf disease on photosynthesis were evaluated using a Li-6400 portable photosynthesis measurement tool (LI-COR Inc., Lincoln, NE, USA) in the inoculation chamber from 9:00 to 11:00 am on three diseased leaves per pot. The standard chamber (window size = 2 × 3 cm) was equipped with a red/blue LED light source with 1200 mol m⁻² s⁻¹ photosynthetically active radiation (PAR), detenting conditions at 28 ± 1 °C, and carbon dioxide concentration of 410 ± 10 μmol (Li et al. 2020). The net photosynthetic rate (µmol/m²·s), stomatal conductance (mol/m²·s), intercellular CO₂ concentration (µmol/mol), and transpiration rate (mmol/m²·s) were also recorded. All plants were harvested 2 weeks after inoculation, and the plant height and root length of each plant were measured. Finally, the plants were oven-dried at 105 °C for 20 min, then 80 °C for 48 h, and their dry weight was determined.

**Statistical analysis**

Data analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) software. Before analysis, the data sets were tested for homogeneity of variance using Levene’s test. The effect of the disease on photosynthesis and biomass was determined using a three-factor analysis of variance (ANOVA). Comparisons between means were performed using Tukey’s honestly significant difference (HSD) at \( P \leq 0.05 \).

**Results**

**Symptoms and incidence of the leaf disease-causing pathogen**

The fungus infection caused blotches on the leaves. Young lesions were ellipsoidal–fusiform, up to 3 mm in diameter, with dark purple margins and an off-white centre (Fig. 1A). Mature lesions were 4 to 8 mm long eye-shaped spots with a light brown center and dark purple edges (Fig. 1B). In the field, the disease incidence on plants and leaves was 100 and 85%, respectively.

**Identification of the pathogen**

A pathogen was isolated from the diseased leaves with an isolation rate of 60%. Four strains (LYZ0587, LYZ0588, LYZ0589, and LYZ0590) were purified and stored in 25% glycerol at −80 °C at the Microbial Strain Bank of Lan-zhou University. The colonies were grey–white with dense mycelia. Their diameters were 1, 3, and 5 cm at weeks 1, 2, and 3, respectively (Fig. 2A). The conidiophores were oblong cylindrical, slightly swollen at the tip, unbranched/branched, not constricted at the septa, slightly conspicuous septa, pale to medium olivaceous brown, smooth, verruculose, slightly to distinctly thickened walls, and up to 1 μm wide. The conidia were light brown with 0 septa;

![Fig. 1](https://example.com/image1.png) *Cladosporium* eyespot symptoms on timothy grass in the field. **A**, Irregularly rounded young lesions, with dark purple margins and off-white centre. **B**, Mature lesions with eye-shaped spots, light brown center, and dark purple edge
12.5 μm × 7.38 μm, 1 septum; 19.36 μm × 8.28 μm, and 2 septa; 27.31 μm × 9.29 μm in the ratio 3:5:2 (Fig. 2B). The fungus was preliminarily identified as *C. phlei* based on the morphological characteristics.

There was a 99% similarity between the isolated pathogen sequences and *C. phlei* (CBS 358.69) in the Genbank. Thus, the fungal pathogen was genetically identified as *C. phlei* (Fig. 3). All the *C. phlei* strains were grouped in one clad, distinguishing them from other *Cladosporium* species (Fig. 3). The gene accession numbers of the pathogen strains in the Genbank are recorded in Table 2.

**Pathogenicity**

The plants inoculated with *C. phlei* developed lesions from the fifth-day post-inoculation with total lesion coverage...
on the leaves occurring 10-day post-inoculation (Fig. 4B), which was consistent with the observations in the fields. The uninoculated plants remained healthy throughout the experimental period. After inoculation, the pathogen reisolated from the diseased spots had an isolation rate of 55%, with LYZ0591 and LYZ0592 strains identified as *C. phlei* through morphological and molecular characterization.

### The effects of the leaf disease on photosynthesis and plant growth

The average net photosynthetic rate and transpiration rate of plants inoculated with the pathogen were 3.42 µmol/m²·s and 0.55 mmol/m²·s, respectively, 14-day post-inoculation and 4.87 µmol/m²·s and 1.25 mmol/m²·s in the controls, respectively. Compared to the control group, the net photosynthetic and transpiration rates decreased by 29.77% and 56%, respectively, following infection with the pathogen (*P* ≤ 0.05). (Fig. 5A, C). The average stomatal conductance of plants inoculated with the pathogen was 24.72 mol/m²·s and 33.82 mol/m²·s in the control group though not statistically significant (Fig. 5B). However, the average intercellular CO₂ concentration on plants inoculated with the pathogen...
was 167.37 µmol/mol and 150.57 µmol/mol in the control group, though not statistically significant (Fig. 5D).

The average plant height and root length of plants inoculated with the pathogen were 12.64 cm and 6.35 cm, respectively, 14-day post-inoculation, and 15.42 cm and 8.69 cm, respectively, in the control group. Compared to the control group, the average plant height and root lengths were 17.96 and 27.02%, significantly lower in the treatment group, respectively ($P \leq 0.05$; Fig. 6A, B). Besides, the average shoot fresh and dry weights per plant in the treatment group were 0.06 g and 0.015 g, respectively, and 0.11 g and 0.028 g in the control group, respectively. Thus, the average shoot fresh and dry weights per plant were decreased by 45.45 and 46.42%, respectively, in the treatment group, which was significantly lower than the control ($P \leq 0.05$; Fig. 6C, D). At the same time, the average root fresh and dry weights per plant in the treatment group were 0.011 g and 0.0031 g, respectively, and 0.014 g and 0.0038 g, respectively, in the control group. Thus, the average root fresh and dry weights in the treatment group decreased by 21.42 and 18.42%,
Discussion

The disease symptoms on the leaves of the timothy grass seedlings 2-week post-inoculation with the pathogen isolated from diseased grass in the field were the same as the symptoms on the timothy grass leaves in the field. This implies that the last step of Koch’s rule was completed, and the pathogen was identified. The leaf disease infecting Timothy grass in Min County was identified as *Cladosporium* eyespot caused by *C. phlei* based on its symptoms, morph- morphology, molecular characterization, and pathogenicity test. Schubert et al. found that within complexes of morphologically similar, closely allied taxa, ITS data are often not sufficient to discriminate species, i.e., the resolution is often too poor, resulting in trees with polytomous structures. However, detailed genetic and morphological examination of the *Cladosporium herbarum* complex demonstrated that a multilocus DNA sequence approach, based on ITS and actin can achieve a better resolution and distinction of closely allied taxa (Schubert et al., 2007). This is consistent with the results of this study. This disease was first identified in Japan in the 1970s (Teruhiko et al. 1975). It is easily distinguished by its symptoms, including eye-shaped spots, light greyish-fawn centers, and purple margins on the leaves (Kang et al. 2019).

*Cladosporium* eyespot infection decreased the shoot fresh and dry weights by 45.45, and 46.42%, respectively. Leaf spot diseases infect timothy causing premature wilt and leaf fall, which reduces the grass yield by 30% and subsequently the seed yields (Kostenko et al. 2012). However, this was not quantitatively characterized on Timothy grass infected by *Cladosporium* eyespot. Still, the losses of Timothy grass yield following inoculation with *C. phlei* were higher than 30%, implying that the disease significantly impacts timothy grass growth. The disease affects photosynthesis by increasing the host or pathogen respiration rate, changing the stomatal conductance, and affecting the photochemical machinery, including the enzymes related to CO$_2$ fixation (Carretero et al. 2011). Herein, the net photosynthetic and transpiration rates were decreased by 29.77 and 56%, respectively, on the diseased leaves. Therefore, it is necessary to quantify the losses under field conditions.

In conclusion, the leaf spot disease infecting timothy grass in Min County, China, is caused by *C. phlei*. The pathogen caused *Cladosporium* eyespot disease, which caused heavy losses to the timothy industry in China; hence, vital to understand the species and biological characteristics of pathogens for disease control. The biological control technique is the best strategy for controlling pasture diseases (Hu et al. 2021). Besides, disease-resistant varieties are crucial, though no disease-resistant varieties have been screened globally.

However, the resistance of timothy grass to *C. phlei* is greatly enhanced in plants already infected with *Epichloë typhina* (So et al. 2012). Therefore, the resistance of *E. typhina* to *C. phlei* is a potential control strategy for *Cladosporium* eyespot infection on timothy. Four polyketide synthase (PKS) genes cluster in the three major clades of fungal PKSs of an ordered fosmid library (So et al. 2012). Among them, the Cppks1 gene is responsible for the biosynthesis of phleichrome in *C. phlei* (So et al. 2015). Multiple integrations of tandem repeat copies of vector DNA at the different chromosomal sites in *C. phlei* facilitate its strain improvement (Kim et al. 2009). In addition, *Epichloë typhina* (anamorph: *Acremonium typhinum*), an endophyte infesting timothy grass, produces cyclo-(L-Pro-L-Leu), which stimulates phleichrome production by *C. phlei*, and cyclo-(L-Pro-L-Phe), which inhibits *C. phlei* growth in culture filtrate. This endophyte produces a cyclic peptide, Epichlicin, which inhibits *C. phlei* conidia germination (Seto et al. 2007). Furthermore, phleichrome has antifungal activity against *E. typhina* in the presence of light (Seto et al. 2005). However, the effects of phleichrome produced by *C. phlei* infesting timothy grass on the horses remain unknown. Therefore, it is necessary to investigate whether endophytes infest timothy grass in Min County and the interaction between *C. phlei*, *Epichloë typhina*, and horses.

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Author contributions BY and YZL designed and performed the experiments. BY analyzed the data. YZL conceived and supervised the project. BY and YZL wrote the paper.

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Availability of data and materials These new generated sequences were uploaded to the GenBank database at the National Center for Biotechnology Information (NCBI), and are available.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This article does not describe any experimental work related to human.

Consent to participate All authors are consent to participate in this manuscript.

Consent for publication All authors are consent for publication in Antonie van Leeuwenhoek.
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