Effects of Timothy Cladosporium Eyespot on Photosynthesis and Biomass

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Research Article

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Abstract

Timothy is a forage mainly grown in Min County, Gansu Province, China. An outbreak of a leaf spot disease infecting timothy grass occurred in Min County in 2020. Therefore, this study investigated the disease incidence in Min County and neighboring counties, and isolated the disease-causing pathogen for morphological and molecular characterization. To precisely diagnose its effects on timothy grass, the growth, photosynthesis, and biomass of timothy seedlings inoculated with the isolated pathogen were investigated. The 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. The disease incidence was 100 and 85% on plants, and leaves, respectively. Morphological and molecular characterization identified the pathogen as *Cladosporium phlei*. The net photosynthetic rate, transpiration rate, fresh shoot weight, and dry shoot weight of timothy seedlings after 14 days of inoculation with the pathogen were decreased by 29.77, 56, 45.45, and 46.42%, respectively. This indicates that cladosporium eyespot disease is an important timothy grass disease in Min County and its environs. Therefore, it is urgent to develop an integrated control strategy to lessen the economic loss.

1 Introduction

Timothy (*Phleum pretense* L.) is a highly palatable grass which produces tender hay and 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 in dogs, cats, rabbits, and rodents (Martineau et al. 1994). Timothy is mainly planted in Min County, Gansu Province in China, occupying 21% of the county's land with 133 hectares in 2021 (Li et al. 2021). The hay is sold to Hong Kong and Inner Mongolia regions of China at 5000–6000 Yuan RMB per ton in Mongolia (Cao 2003). Min County is a less developed mountainous area located south of Gansu. Timothy is the key source of income for the local farmers (Qi 2012). Timothy was initially 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 suitable for timothy growth in Min County (Hoglind et al. 2001). 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).

In 2020, a severe leaf disease affected the timothy grass in Min County, affecting its yields, nutrition quality, aesthetic value and consequently reducing the famers income. This disease remains unknown, and its influence on timothy grass growth has not been defined. Therefore, the objective of this study is to establish what this disease is and its importance to timothy growth.

2 Materials And Methods

2.1 Field survey

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 observed. Min County 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.

Five sites per county were selected using a handheld global positioning system. Five fields ranging from 1,000 to 2,500 m^2 per site were arbitrarily selected for the incidence survey. The number of healthy and potentially infected plants exhibiting the leaf disease symptoms, including eye-shaped spots with a light brown center and dark purple edge, were recorded in each field. Next, the disease incidence was calculated as the proportion of symptomatic plants over the total number of plants recorded. The symptoms were observed and recorded during the early, middle, and late stages of disease

development. In addition, qualitative checks across the fields were done to give an overall impression of the incidence and effects of this disease. Five plants with diseased leaves were sampled in each field by cutting them at the base of their stems and individually packaged for pathogen isolation.

2.2 Isolation and identification

Diseased leaves collected from the fields were thoroughly washed under running tap water, surface sterilized with 75% alcohol for 30 s followed by 1 min in1% sodium hypochlorite, rinsed in three changes of sterile distilled water, dried on a sterile paper, and sectioned into 0.5 mm sections. The sections were cultured on Potato Dextrose Agar (PDA) and incubated in the dark at 25°C for 10 to 15 days (Xu et al. 2019). Fungal mycelia on the leaf sections were further subcultured on PDA media to obtain pure isolates. The pure isolates were morphologically characterized based on the conidia shape and color. In addition, the conidiophores on two-week-old cultures were observed under a light microscope (Nikon ECLIPSE *Ti*, Japan). The sizes of 50 conidia and conidiophores were also measured. Finally, the colony, conidia, and conidiophore images were taken with a digital camera (Leica D-LUX7). The total genomic DNA of pure colonies was extracted using the Ezup Fungal DNA Kit (Sangon Biotech), following the manufacturer's protocol for molecular characterization. The extracted genomic DNA was amplified in a 2720 Thermal Cycler (Applied Biosystems) in a total volume of 25 ml with ACT primers; 512F(5'-ATGTGCAAGGCCGGTTTCGC-3')/783R(5'-TACGAGTCCTTCTGGCCCAT-3') and ITS1(5'-TCCGTAGGTGAACCTGCGG-3')/ITS4(5'-TCCTCCGCTTATTGATATGC-3') as described by Bensch et al. (2012). The PCR products were visualized on 1.0% agarose gels, and the positive products were purified and sequenced by 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) method using MEGA5.0 software.

2.3 Pathogenicity tests

Timothy grass seeds obtained from farmers in Min County were sown in pots (height 15 cm, caliber 10 cm) in 2021. Each pot contained 1.5 kgs of double-autoclaved soil at 121°C for 1 h with a 3-day interval between sterilization. Seven seeds were sown per pot and maintained in a greenhouse at 25/20°C day/ night temperatures and average relative humidity of 70%. After 3 weeks, conidial suspension (1× 10^6 conidia ml-1) was sprayed on the seedlings, with seedlings sprayed with sterile water serving as controls.

The presence of the leaf disease symptoms on the seedlings was monitored daily. The disease incidence was investigated when the symptoms matched those under field conditions. Lesions on 10 leaves per pot were used to reisolate and confirm the pathogen based on colony and spore morphology.

2.4 Effect of the leaf disease on photosynthesis and plant growth

In addition, plants in six treatment and six control pots were covered with black plastic bags for 48 h, then randomly arranged in an inoculation chamber. The chamber (measure window = 2 × 3 cm) was equipped with a red/blue LED light source, with photosynthetic active radiation (PAR) of 1200 mol $m^{-2}s^{-1}$, detesting conditions at 28 ± 1°C, and air carbon dioxide concentration of 410 ± 10 μmol CO_2 mol^{-1} (Li et al. 2020). The leaf disease effects on photosynthesis were evaluated using a Li-6400 portable photosynthesis measurement tool (LI-COR Inc., Lincoln, NE, USA) from 9:00 to 11:00 am on three diseased leaves per pot. The net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) were also recorded. All plants were harvested two weeks after inoculation. After harvest, the plant height and root length of each plant were measured. In addition, the plants were oven-dried at 105°C for 20 min, and 80°C for 48 h, and their dry weight was determined.

2.5 Statistical analysis

All 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 $P \leq 0.05$.

3 Results

3.1 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. 1-A). Mature lesions were eye-shaped spots, 4 to 8 mm long, with a light brown center, and dark purple edge (Fig. 1-B). The incidence rate of the infection on plants was 100%, and 85% on leaves.

3.2 Identification of the pathogen

A fungus was isolated from 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 Lanzhou University. The colonies were gray-white with dense mycelia. Their diameters were 1, 3, and 5 cm in diameter at weeks 1, 2, and 3, respectively (Fig. 2-A). 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 thickened to distinctly thickened walls, and up to 1 µm wide. The conidia were light brown with 0 septa, 12.5 µm × 7.38 µm, 1 septum, 19.36 µm × 8.28 µm, 2 septa, 27.31 µm × 9.29 µm; the ration of these three conidia was 3:5:2 (Fig. 2-B). The fungus was preliminarily identified as *C. phlei* based on these morphological characteristics.

There was a 99% similarity between the ITS and ACT sequences to *C. phlei* (CBS 358.69) in the Genbank. Thus, the fungal pathogen was genetically identified *C. phlei* (Fig. 3). All the *C. phlei* strains grouped in one clad, distinguishing them from other *Cladosporium* species (Fig. 3).

3.3 Pathogenicity

The plants inoculated with *C. phlei* developed lesions from the fifth-day post-inoculation with total lesion coverage on the leaves ten days post-inoculation (Fig. 4-B), the same as in the fields. The uninoculated plants remained healthy throughout the experimental period. A fungus was reisolated from the disease spots after inoculation at an isolation rate of 55%, with LYZ0591 and LYZ0592 strains identified as *C. phlei* by morphology and molecular methods.

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

The net Pn and Tr in plants inoculated with the pathogen 14 days post-inoculation were decreased by 29.77% and 56%, respectively, significantly lower than the control ($P \leq 0.05$). (Fig. 5-A and C). The Gs was slightly lower than the control, though not statistically significant (Fig. 5-B). However, the intercellular CO_2 concentration was slightly higher than the control, though not statistically significant (Fig. 5-D).

The plant height and root length in plants inoculated with the pathogen were significantly lower and shorter than the control ($P \leq 0.05$; Fig. 6-A and B), reduced by 17.96% and 27.02%, respectively, 14 days post-inoculation. Besides, the shoot fresh and dry weights in plants inoculated with the pathogen were decreased by 45.45% and 46.42%, respectively, 14 days post-inoculation, significantly lower than the control ($P \leq 0.05$; Fig. 6-C and D). At the same time, the root fresh and dry weights in plants infected with the pathogen were decreased by 21.42% and 18.42%, respectively, 14 days after inoculation, which was significantly lower than the control ($P \leq 0.05$; Fig. 6-E and F).

4 Discussion

The leaf disease infecting Timothy grass in Min County was identified as cladosporium eyespot caused by *C. phlei* based on its symptoms, morphological and molecular characteristics, and the pathogenicity test. This disease was firstly identified in Japan in the 1970s (Teruhiko et al. 1975). It is easily distinguished by its symptoms, which include eye-shaped spots, light grayish-fawn centers, and purple margins on the leaves (Kang et al. 2019). Up to 15 variants of this disease on timothy grass have been reported in China, including stripe rust (*Puccinia striiformis*) and cladosporium eyespot (*C. phlei*) in eastern Gansu (Nan 1990), leaf spot *(Hadrotrichum phragmiticolum*) and leaf blight (*Brachsporium phragmitis*) in Hebei, and net spot (*Helminthosporium dictyoides*) in Jilin (Jiang 1959). However, only cladosporium eyespot has been identified in Min County. Therefore, further studies investigating the possibility of other variants in Min County are needed.

Cladosporium eyespot decreased the shoot fresh and dry weights by 45.45, and 46.42%, respectively. Many leaf spot diseases on Timothy cause premature wilting, and leaf fall reduces grass yield by 30% and, subsequently, seed yields (Kostenko et al. 2012). However, this was not quantitatively characterized on Timothy grass infected by *Cladosporium* eyespot. This study revealed that the losses in Timothy plants inoculated with *Cladosporium* eyespot are higher than 30%, implying that the disease significantly impacts timothy grass growth. It affects photosynthesis by (i) increasing the host or pathogen respiration rate, (ii) changing the stomatal conductance, and (iii) affecting the photochemical machinery, including the enzymes related to CO_2 fixation (Carretero et al. 2011). Here, Pn, Gs, and Tr on diseased leaves were decreased by 29.77% and 56%. Therefore, it is necessary to quantify the loss under field conditions.

In conclusion, the findings presented here confirm that *C. phlei* has already become established in some regions of Min County, China and that this disease is a heavy losses to the Timothy industry in China. It is vital to understand the species and biological characteristics of pathogens for disease control. The best strategy for controlling pasture diseases is the biological control technique(Hu et al. 2021). Besides, the use of disease-resistant varieties is crucial, though no disease-resistant varieties have been screened globally.

The resistance of timothy grass to *C. phlei* is greatly enhanced in plants already infected with *Epichloe typhina* (So et al. 2012). Therefore, the resistance of *E. typhina* to *C. phlei* is a potential control strategy of cladosporium eyespot 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, *Epichlöe 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 kind of cyclic peptide, Epichlicin, which inhibits *C. phlei* conidia germination (Seto et al. 2007). Furthermore, phleichrome has antifungal activity against *E. typhina* in the light (Seto et al. 2005). However, the effects of phleichrome produced by *C. phlei* infecting timothy grass on the horses remain unknown. Therefore, it is necessary to investigate further if endophytes are infesting timothy grass in Min County and if they are there, the interaction between *C. phlei*, *Epichlöe typhina*, and horses.

Declarations

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Authors' contributions

BY and Y-ZY designed and performed the experiments. BY analyzed the data. Y-ZL conceived and supervised the project. BY and Y-ZL wrote the paper.

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Availability of data and material

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

Ethics declarations

Conflicts of Interest

The authors declare no conflicts of interest.

Ethical standards

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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Table

Table 1

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
					ITS	ACT	
Cladosporium acalyphae	CBS 125982	*Acalypha australis*	South Korea	H.D.Shin	HM147994	HM148481	(Bensch et al. 2010)
Cladosporium allicinum	CBS 121624	*Hordeum vulgare*	Belgium	J.Z.Groenewal	EF679350	EF679502	(Schubert et al. 2007)
Cladosporium angustisporum	CBS 125983	*Alloxylon wickhamii*	Australia	B.A.Summerell	HM147995	HM148482	(Bensch et al. 2010)
Cladosporium asperulatum	CBS 126340	*Protea susannae*	Portugal	—	HM147998	HM148485	(Bensch et al. 2010)
Cladosporium australiense	CBS 125984	*Eucalyptus moluccana*	Australia	B.A.Summerell	HM147999	HM148486	(Bensch et al. 2010)
Cladosporium basiinflatum	CBS 822.84	*Hordeum vulgare*	Germany	—	HM148000	HM148487	(Bensch et al. 2010)
Cladosporium chubutense	CBS 124457	*Pinus ponderosa*	Argentina	A.Greslebin	FJ936158	FJ936165	(Schubert et al. 2009)
Cladosporium colocasiae	CBS 386.64	*Colocasia esculenta*	Taiwan	K.Sawada	HM148067	HM148555	(Bensch et al. 2010)
Cladosporium delicatulum	CBS 126344	*Tilia cordata*	Germany	K.Schubert	HM148081	HM148570	(Bensch et al. 2010)
Cladosporium exile	CBS 125987	*Phyllactinia guttata*	USA	D.Glawe	HM148091	HM148580	(Bensch et al. 2010)
Cladosporium funiculosum	CBS 122129	*Vigna umbellata*	Japan	—	HM148094	HM148583	(Bensch et al. 2010)
Cladosporium gamsianum	CBS 125989	*Strelitzia* sp.	South Africa	W.Gams	HM148095	HM148584	(Bensch et al. 2010)
Cladosporium herbarum	CBS 121621	*Hordeum vulgare*	Netherland	—	EF679363	EF679516	(Schubert et al. 2007)
Cladosporium inversicolor	CBS 401.80	*Triticum aestivum*	Netherland	—	HM148101	HM148590	(Bensch et al. 2010)
Cladosporium perangustum	CBS 125996	*Cussonia* 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.

Figures

Figure 1

Field symptoms. A, The young lesions are irregularly rounded, with dark purple margins and off-white centre. B, Mature lesions are eye-shaped spots with light brown center and dark purple edge.

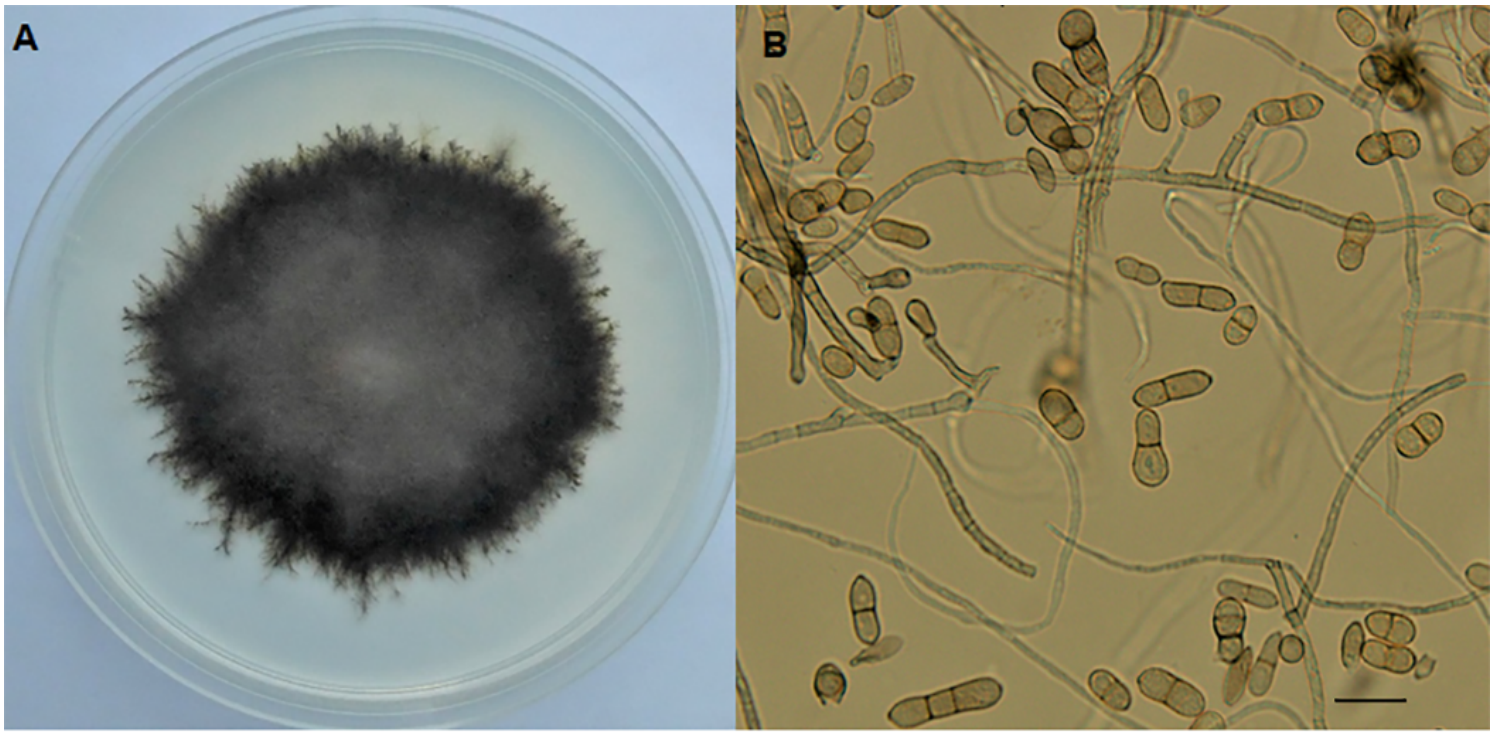

Figure 2

Morphological characteristics of the fungus isolated from timothy. A, morphological characteristics of colonies at 3 weeks. B, conidia and conidiophore. Scale bars in D is 20μm.

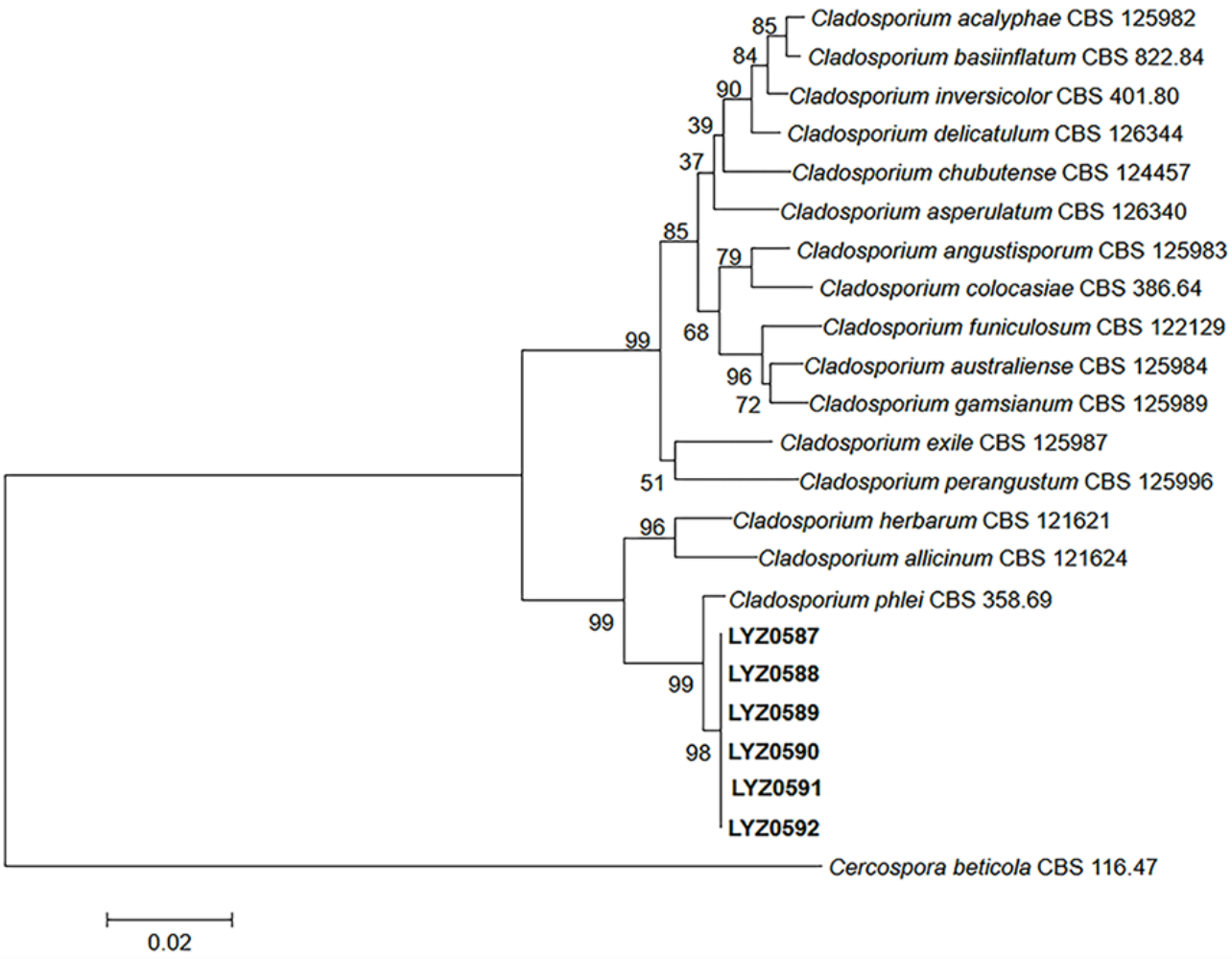

Figure 3

A neighbor-joining phylogeny of the concatenated partial sequences of ITS and ACT gene regions of Cladosporium isolates used in this study. Cercospora beticola was used as outgroup.

Figure 4

Timothy mature seedlings inoculated with C. phlei after two weeks postinoculation. A, The seedling on the right is inoculated with C. phlei, and the seedling on the left is the control. B, Symptom of inoculated plant.

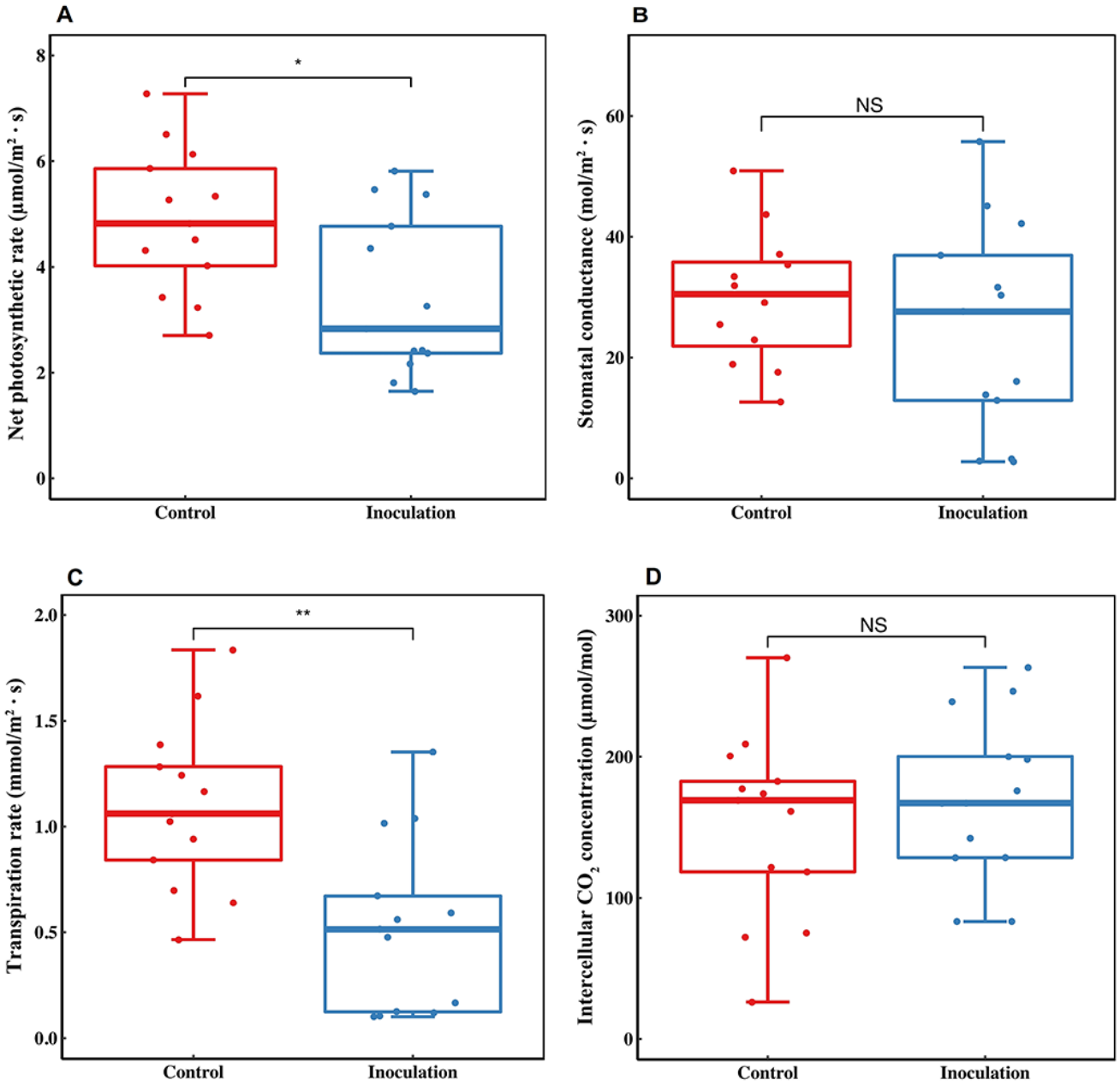

Figure 5

Comparison of photosynthetic parameters per plant between inoculated timothy and controls treated with sterilized water. A, The net photosynthetic rate (Pn) of inoculated and control. B, The stomatal conductance (Gs) of inoculated and control. C, The transpiration rate (Tr) of inoculated and control. D, The intercellular CO2 concentration of inoculated control. Values are mean ± standard error (SE), with bars indicating SE. *Significant difference at P ≤ 0.05 (independent t test).

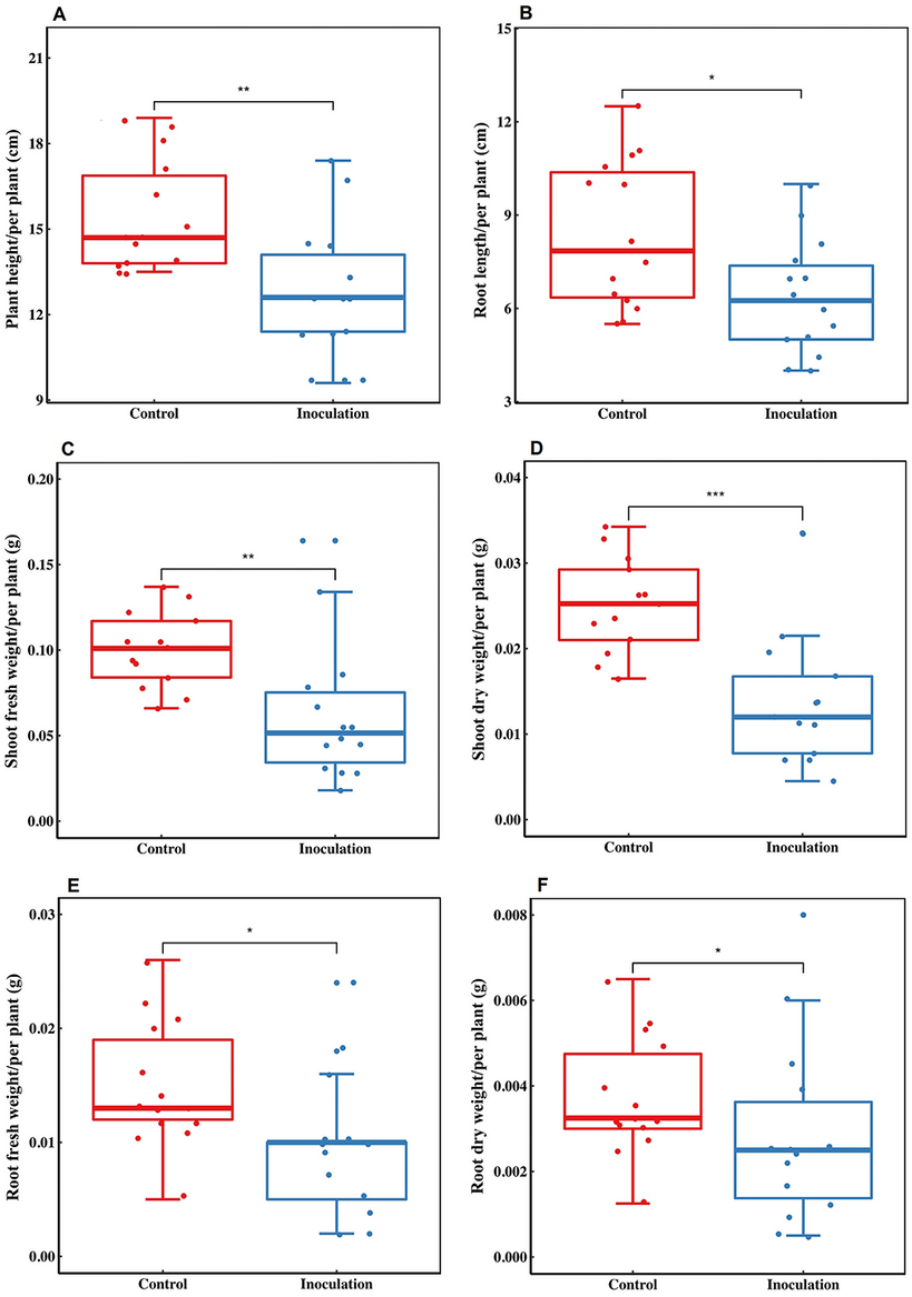

Figure 6

Comparison of biomass parameters per plant between inoculated timothy and controls treated with sterilized water. A, The plant height of inoculated and control. B, The root length of inoculated and control. C, The shoot fresh weight of inoculated and control. D, The shoot dry weight of inoculated and control. E, The root fresh weight of inoculated and control. F, The root dry weight of inoculated and control. Values are mean ± standard error (SE), with bars indicating SE. *Significant difference at $P \leq 0.05$ (independent t test).