Identification, Pathogenicity, and Fungicide Sensitivity of *Ascochyta caulina* (Teleomorph: *Neocamarosporium calvescens*) Associated with Black Stem on Quinoa in China

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Abstract

Quinoa black stem is a new disease that affects the stems of quinoa plants and is more likely to develop under cool conditions (15 to 25°C, RH = 55 ± 2%). The typical symptoms include the formation of black necrotic lesions on the stem, which can completely wrap around the stem, causing lodging and blanking (development of 'empty' and sterile grain on the panicle). Furthermore, the pycnidia form small round protrusions on the surface of the lesions. Phylogenetic analysis revealed that representative isolates LMHS-3 and LMHS-5 were closely related to *Ascochyta caulina* (teleomorph: *Neocamarosporium calvescens*). Comprehensive morphological and molecular characterizations confirmed *A. caulina* as the pathogen that caused quinoa black stem. *A. caulina* mainly infected quinoa stems and could produce many pycnidia, but it rarely infected quinoa leaves. Pathogenicity testing showed that the most suitable temperature for the

onset of quinoa black stem was from 15 to 25°C. When the temperature was increased above 30°C, the conidial germination of *A. caulina* became malformed, and when the temperature was decreased below 5°C, mycelium growth of *A. caulina* became extremely slow; thus, both extreme high and low temperatures affected the pathogenicity of *A. caulina*. Mancozeb and azoxystrobin fungicides were revealed to have had the strongest inhibitory effects on the conidial germination of *A. caulina*, and in some cases caused malformations in conidial germination. Tebuconazole and difenoconazole had the strongest inhibitory effects on *A. caulina* mycelial growth and less on the effects on the conidial germination. The results of the present study provide a basis for the recognition and management of quinoa black stem.

Keywords: quinoa, fungal disease, morphology, conidia, teleomorph

Quinoa (*Chenopodium quinoa*) is a dicotyledonous annual plant in the family Amaranthaceae (Maughan et al. 2007). It is currently being grown as a new food crop in more than 90 countries across South America, Europe, Oceania, and Asia (Singh et al. 2016), among which Bolivia, Peru, Ecuador, and Chile are the main exporters (Pellegrini et al. 2018). Since 2014, the large-scale cultivation of quinoa has begun in Shanxi, Qinghai, Gansu, and Hebei provinces of China, covering an area of ~3,333 ha, thus showing an increasing trend in development (Ren et al. 2015).

With quinoa cultivation becoming more widespread, the occurrence of quinoa diseases in China, such as quinoa downy mildew and Cercospora leaf spot, have become increasingly serious and threaten the development of the quinoa industry (Yin et al. 2018, 2019). In 2017, a new disease of quinoa stems was identified in plantations in Jingle, Ningwu, Wutai, and Shanyin counties of Shanxi Province, China. This disease was first observed during the flowering stage of quinoa and the lengths of the lesions were from 0.3 to 1.2 cm. In the early stages of this disease, the lesions appeared pale, and the margins between the lesions and healthy tissue were clear. In the later stages, the main lesions became blackened and were accompanied by numerous small black spots. In severe cases, the plants exhibited lodging, leaf yellowing, leaf shedding, and blanking (development of 'empty' and sterile grains on the panicle). In 2017, we found that disease incidence was ~80%, and the yield was consequently reduced by ~45% in the quinoa fields where the disease was most severe in Jingle County, Shanxi Province.

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Similar necrotic lesions on the leaves and stems of Chenopodiaceae plants have also been reported (van der Aa and van Kesteren 1979). Typically, the *Ascochyta* leaf spot and stem lesions on quinoa caused by *Ascochyta hyalospora* have light spots of indefinite areas on the leaves, and stem necroses (Boerema et al. 1977; Li et al. 2017). At the later stages of the disease, *Ascochyta* leaf spots of quinoa become dry and the leaves fall off (Li et al. 2017). Black stem disease of quinoa, investigated in this study, mainly results in black necrotic lesions on the stems and rarely on the leaves.

Ascochyta leaf spot and stem necroses on various species of Chenopodiaceae and *Atriplex* have already spread in Europe and Siberia by *A. hyalospora* and *A. caulina* (Boerema et al. 1977, van der Aa and van Kesteren 1979). van der Aa and van Kesteren (1979) reported that *A. caulina* infected eight species of *Atriplex* (*A. hortense*, *A. lacinata*, *A. littoralis*, *A. nitens*, *A. patula*, *A. hastata*, *A. hortensis*, and *A. oblongifolium*) and eight species of *Chenopodium* (*C. album*, *C. bonus-henricus*, *C. glaucum*, *C. hybridum*, *C. murale*, *C. viride*, *C. suecicum*, and *C. vulvaria*). Boerema et al. (1977), de Gruyter et al. (2009), and van der Aa and van Kesteren (1979) reported that *A. caulina* and *A. hyalospora* had similar morphologies. There are multiple synonyms on *Chenopodium* plants caused by these species that are similar (van der Aa and van Kesteren 1979), so the two species are often confused.

In 2003, Dřímalková detected *A. caulina* in quinoa seeds from the Czech Republic, but they did not report that *A. caulina* could infect quinoa (Dřímalková 2003). Presently, there are no definitive reports that *A. caulina* can infect quinoa. There is confusion in the research concerning *Ascochyta* leaf spot of quinoa. The confusion stems from the inaccurate classification of the pathogens and a lack of previous systematic research. The sexual stage of *A. caulina* is called *Pleospora calvescens* (Boerema et al. 1987). *P. calvescens* has multiple synonyms, including *P. chenopodii*, *P. halimiones*, *Pyrenophora calvescens*, *Chaetoplea calvescens*, *Leptosphaeria calvescens*, and *Phloeospora calvescens* (de Gruyter et al. 2009). As fungal classification techniques have developed, the taxonomic status of *Pleospora calvescens* has changed greatly. Recent taxonomic revisions based on LSU, SSU, and ITS gene analyses have placed *P. calvescens* in the genus *Neocamarosporium* Crous & Wingfield and identified it

as *N. calvescens* (Ariyawansa et al. 2015; Wanasinghe et al. 2017). The classification of the *Neocamarosporium* (Crous & Wingfield) genus was established in 2014 and comprises 15 species, including *N. betae*, *N. chenopodii*, *N. obiones*, and *N. calvescens* (Crous et al. 2014; Grum-Grzhimaylo et al. 2016; Papizadeh et al. 2018; Wanasinghe et al. 2017). *N. calvescens* (anamorph: *A. caulina*) has recently been reported in countries such as Germany and the Netherlands.

Currently, there are no systematic reports on quinoa black stem, as it is considered a new emerging disease of quinoa in China. This study described the symptoms of quinoa black stem, systematically characterized the fungus causing quinoa black stem, and determined the sensitivity of this pathogen to common fungicides and different temperatures, to provide a basis for the recognition and prevention of the disease.

Materials and Methods

Collection and isolation of samples. From flowering to the stage of maturity in 2017, typical black stems were collected from six quinoa plantations in Jingle County, Shanxi Province, China. All stem samples were initially examined using an Olympus BX53 microscope (Olympus, Japan) to further select test stem samples for pathogen isolation. For stem samples showing disease symptoms, the pathogens were directly picked from the lesions using a stereomicroscope and cultured on potato dextrose agar (PDA) (Solarbio, China) at 25°C for 5 to 7 days until colony formation (Sun et al. 2003). The colony was cultured on PDA at 25°C for 20 days until pycnidia formed. The resulting colonies were further purified by culturing single conidia according to the previously described procedure (Bai et al. 2015). Pycnidia were collected and ground, then diluted to a low concentration with sterilized water. The conidia dilutions were spread on PDA to generate discrete colonies. Pure fungi were obtained and stored in a refrigerator at 4°C. Colony characteristics and microscopic observations of all isolates were examined to select representative isolates.

Morphological characterization of isolates. The representative isolates were cultured on PDA (Solarbio, China) at 25°C for 20 days, and the colony morphologies were observed. The representative isolates were inoculated on cellophane-coated PDA for culture at 25°C. Pycnidia formation was observed using an Olympus BX53 microscope (Olympus, Japan).

Specimens with typical small black spots (pycnidia) were selected. These specimens were cut into small pieces (0.5 × 0.5 cm). Tissue samples (2 ml) were embedded in a tissue freezing medium (Leica, Germany). A Leica CM1950 cryostat was used to section the tissues to a thickness of 6 to 8 µm at -20°C. Microscopic features such as pycnidia and conidia were observed and measured using an Olympus BX53 microscope.

In order to view the details of the pycnidia ultrastructures, electron microscopy was performed using a JSM-6390 (JEOL Ltd., Japan) scanning electron microscope. Specimens with small black spots were cut into small pieces of ~1 mm³, immersed in a 2.0% glutaraldehyde solution (pH 7.2), and placed in a refrigerator at 4°C for 12 h. After fixation, the samples were rinsed with 0.15% glutaraldehyde phosphate buffer and then dehydrated consecutively with 40, 70, 90, and 100% ethanol for 15 min each time. After dehydration, the ethanol was replaced with amyl acetate, the samples were placed in a critical point drier, immersed in liquid carbon dioxide, and heated to above the critical temperature point of 31.4°C to dry by evaporation. After drying, the pycnidia were cut open, and a JEOL JEE-420 (JEOL Ltd., Japan) vacuum evaporator was used for gold sputtering. The ultrastructures of the pycnidia were then observed using a JSM-6390 (JEOL Ltd., Japan) scanning electron microscope.

Effects of temperature on mycelium growth and conidia germination. Representative isolates were inoculated onto PDA and placed in an incubator with a temperature gradient of 0, 5, 10, 15, 20, 25, 30, and 35°C. Colony diameters were measured after 20 days when the pycnidia were mature.

Pycnidia were collected and ground from the representative isolates that were incubated on the PDA at 25°C for 20 days. A conidia

suspension was prepared to a concentration of 1×10^5 conidia/ml with sterile water. This suspension (100 µl) was inoculated onto a PDA-coated slide and placed in a desiccator with a relative humidity (RH) of 75% (saturated NaCl saline solution). The desiccator was used to provide an enclosed environment to maintain humidity. The desiccator was placed in an incubator and set to a temperature gradient of 0, 5, 10, 15, 20, 25, 30, and 35°C. After 12 h, the conidia germination was examined by microscopy, and the germination rate was calculated. Each treatment was repeated three times, and 500 conidia were observed in each microscope slide prepared (Zang and Huang 2007).

A HOBO Pro V2 temperature/RH data logger (Onset, U.S.A.) was used to monitor the temperatures and RH in the incubator. The temperatures fluctuated within a range of $\pm 0.5^\circ\text{C}$, and the RH fluctuated within a range of $\pm 2.0\%$.

Molecular characterization of the isolates. Mycelia were cultured for 20 days, collected, and then ground in liquid nitrogen. DNA was extracted using an Ezup column fungi genomic DNA purification kit (Sangon Biotech [Shanghai] Co., Ltd., Shanghai, China). The LSU, SSU, and ITS sequences were amplified using three pairs of primers (Table 1). The PCR amplification procedures for the LSU, ITS, and SSU were as follows: predenaturation at 95°C for 5 min, denaturation at 95°C for 90 s, annealing for 90 s, 35 cycles of extension at 72°C for 1 min, and the final extension at 72°C for 10 min. The annealing temperature for the LSU and ITS sequences was 55°C, and the annealing temperature for the SSU was 48°C (Wanasinghe et al. 2017). The PCR products were determined using 1% agarose gel electrophoresis, and the products were recovered using a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). The PCR products were sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing, and the sequences were obtained and uploaded to GenBank (Table 2). Using *Phoma drobnjakensis* (CBS 269.92) as an outgroup, a phylogenetic tree was constructed based on the LSU, SSU, and ITS gene sequences by the neighbor-joining method using PAUP* 4.0b10 software, with 1,000 repetitions (Swofford 2003).

Pathogenicity analysis. *Pathogenicity on detached stems and leaves.* Stem segments (~10 cm long and ~2 cm in diameter) of *C. album*, *C. bryoniifolium*, and *C. quinoa* were collected from the quinoa fields at the flowering stage. The detached stems were sealed at both ends with Parafilm. Before inoculation, the stems were rinsed with sterile water several times and then air-dried. The conidia suspension (200 µl) as described previously was drip dropped onto the unwounded stems of the quinoa. The control group was inoculated similarly with sterile water. Ten stems of quinoa, *C. album*, and *C. bryoniifolium* were inoculated for each treatment. The inoculated stems were placed in a climate chamber with a temperature gradient of 10, 15, 20, 25, 30, and 35°C and RH = $75 \pm 2.0\%$. The symptoms were monitored and recorded over 6 to 10 days, until the experiment was complete. The length of the lesions was measured at 10 days postinoculation (dpi).

Detached leaves were collected at the flowering stage of *C. album*, *C. bryoniifolium*, and *C. quinoa* (white quinoa: Jingli No. 1, red quinoa: Jingli No. 3) from the quinoa fields. Before inoculation, detached leaves were rinsed with sterile water several times and then air-dried. For inoculation of the conidia, the 100 µl of suspension at a concentration of 1×10^5 conidia/ml was drip dropped onto the upper side of each leaf, and then the petiole was covered with sterilized cotton. Control leaves were inoculated in parallel using sterile water. After this, the inoculated leaves were incubated in climate chambers with a temperature gradient of 10, 15, 20, 25, 30, and 35°C, and RH = $75 \pm 2.0\%$, with 12 h of intermittent light. Each treatment was applied to three leaves and repeated five times. The symptoms were monitored and recorded over 3 to 8 days, until the experiment was complete. The lesion size of the leaf spot was measured 8 dpi.

Pathogenicity on intact stems and leaves. Healthy *C. quinoa* plants were inoculated on the stems at the flowering stage, which were cultivated in the greenhouse from seed (fluorescent cycle of 12 h light/12 h dark at 22°C). To investigate the pathogenicity of

the fungal isolates outside, *C. quinoa* from the greenhouse were transplanted into pots and inoculated with 200 µl of a conidial spore solution prepared as previously described. Then the inoculated plants were placed outdoors in Taiyuan, Shanxi Province, China,

from late October to November of 2019. The symptoms were recorded over 6 to 15 days. Pathogens were isolated again from the lesions after the development of disease and compared with the inoculated isolates (Lupien et al. 2017). Intact leaf inoculations

Table 1. Primers used to amplify LMHS-3 and LMHS-5 of *Ascochyta caulina* in this study

Primer pair	Sequence 5' to 3'	Annealing temperature	Amplified regions
ITS1	TCCGTAGGTGAACCTGCG	55°C	ITS
ITS4	TCCTCCGCTTATTGATATGC		
LROR	ACC CGC TGA ACT TAA GC	55°C	LSU
LR7	TAC TAC CAC CAA GAT CT		
NS1	GTAGTCATATGCTTGTCTC	48°C	SSU
NS4	CTTCCGTC AATTCTTTAAAG		

Table 2. Isolates and GenBank accession numbers used for phylogenetic analysis

Species	Strain number	Previous name	Host plant	GenBank accession number		
				LSU	SSU	ITS
Not yet determined	LMHS-3	-	<i>Chenopodium quinoa</i>	MN064796	MN064798	MN065158
	LMHS-5	-		MN064797	MN064799	MN065159
<i>Neocamarosporium calvescens</i>	CBS 246.79	<i>Pleospora calvescens</i>	<i>Atriplex hastata</i>	MH872973	EU754032	KY940774
<i>N. chenopodii</i>	CBS 344.78	<i>P. chenopodii</i>	<i>Atriplex hastata</i>	MH872910	EU754033	MH861148
<i>N. obiones</i>	CBS 432.77	<i>P. halimiones</i>	<i>Halimione portulacoides</i>	MH872851	JF740096	MH861086
<i>N. betae</i>	CBS 109410	<i>Phoma betae</i>	<i>Beta vulgaris</i>	MK249662	MK249665	KY940790
	CBS 523.66			MH870520	EU754080	FJ426981
<i>N. lamiacearum</i>	ICMP 10945			MK249661	MK249664	MK249658
	MFLUCC 17-560	-	<i>Lamiaceae</i> spp.	MF434279	MF434367	MF434191
<i>N. salicorniicola</i>	MFLUCC 17-0750	-	-	MF434280	MF434368	MF434192
	MFLUCC 15-0957	-	<i>Salicornia</i> spp.	MF434281	MF434369	MF434193
<i>N. korfii</i>	MFLUCC 17-0745	-	-	MF434278	MF434366	MF434190
<i>N. salsolae</i>	MFLUCC 17-0827	-	<i>Salsola</i> spp.	MF434283	MF434371	MF434195
	MFLUCC 17-0826		<i>Salsola</i> spp.	MF434282	MF434370	MF434194
<i>Phoma drobnjakensis</i>	CBS 269.92	<i>Subplenodomus drobnjakensis</i>	<i>Eustoma exaltatum</i>	JF740285	JF740100	JF740211

Table 3. Concentration of the seven fungicides used in this study

Fungicide	Concentration on conidia germination (µg/ml)	Concentration on mycelia growth (µg/ml)
Carbendazim	350, 300, 250, 200, 150	5, 4, 3, 2, 1
Tebuconazole	350, 300, 250, 200, 150	0.1, 0.05, 0.025, 0.01, 0.0005
Prochloraz	120, 100, 80, 70, 60	8, 4, 2, 1, 0.5
Difenoconazole	100, 90, 80, 70, 60	0.1, 0.05, 0.025, 0.01, 0.0005
Thiophanate-methyl	80, 70, 60, 50, 40	6, 3, 1.5, 1, 0.5
Azoxystrobin	20, 10, 5, 2.5, 1	1, 0.5, 0.25, 0.125, 0.1
Mancozeb	10, 8, 5, 2.5, 1	1, 0.5, 0.25, 0.125, 0.1

Fig. 1. Typical symptoms of quinoa black stem on *Chenopodium quinoa* in the field. **A**, New developed diamond shaped lesions on quinoa stem. Arrows indicate the pycnidia on the stems. **B** and **C**, Pycnidia on the infected stems of white quinoa (**B**) and red quinoa (**C**). **D**, Lodging symptoms of quinoa black stem. **E**, Pycnidia on a dead stem of quinoa.

were performed at the flowering stage of *C. album*, *C. bryoniifolium*, and *C. quinoa* cultivated in the greenhouse. A conidia suspension (100 μ l), at a concentration of 1×10^5 conidia/ml, was inoculated onto the upper side of each leaf. The control group was inoculated with sterile water. Three replicates were performed

for the inoculation. The symptoms were monitored and recorded over 5 to 10 days until the experiment was complete. Pathogens were isolated again from the lesions after the development of the disease and compared with the inoculated isolates (Lupien et al. 2017).

Fig. 2. Morphologies of the representative isolates of quinoa black stem. **A**, Views of the surface (I) and the bottom (II) of the colony from the PDA plate of the representative isolate. **B**, Formation of pycnidia and the adjacent mycelia connection and fusion (I), further gathered around the center (II), and becoming pycnidial primordia (III); pycnidial primordia became subglobose (IV) and further expanded (V) until primordia matured (VI). **C**, Morphological characteristics of the pycnidia and conidia (I), and the ultrastructure of the pycnidia (II).

Pathogenicity on roots. Before sowing, the seeds of the *C. quinoa* and *C. album* were rinsed with sterile water several times and then air-dried. Seeds were sown into pots with sterilized peat moss. Pots were placed in a climate chamber (fluorescent cycle of 12 h light/12 h dark, RH = 45 ± 2.0%, at 22°C). Plants were grown for 2 weeks and were approximately 10 to 12 cm tall when they were inoculated. For inoculation, each plant was drench-treated with 100 ml of the conidia suspension as described previously. The controls were inoculated with sterile water. To examine

the underground symptoms, soil attached to the roots was gently washed, then photographed 2 weeks postinoculation.

Sensitivity of mycelial growth and conidia germination to seven fungicides. Azoxystrobin (97.50%), difenoconazole (97%), and tebuconazole (97%) were each dissolved in methanol to prepare 1.0×10^4 µg/ml stock solutions. Mancozeb (85%), thiophanate-methyl (95%), and prochloraz (95%) were each dissolved in acetone to prepare 1.0×10^4 µg/ml stock solutions. Carbendazim (96%) was dissolved in 0.1 mol/liter dilute hydrochloric

Fig. 3. Phylogenetic tree of *Neocamarosporium calvescens* (anamorph: *Ascochyta caulina*) isolates (LMHS-3 and LMHS-5) and its related isolates based on LSU, SSU, and ITS sequences using the neighbor-joining method. *Phoma drobnjakensis* was used as an outgroup. Bootstrap values (expressed as percentages of 1,000 replications) were shown at the major branching points. The scale bar represents 0.1 substitutions per nucleotide.

acid to prepare a 1.0×10^4 $\mu\text{g/ml}$ stock solution (Hou et al. 2014; Huang et al. 2016; Xu et al. 2016).

The seven fungicidal stock solutions were diluted into serial dilutions using sterile water and added to PDA at $\sim 50^\circ\text{C}$ to prepare the plates and slides (Table 3). Preliminary testing showed that the volume fractions of the methanol, acetone, or hydrochloric acid were less than 0.25%; this did not affect the mycelial growth or spore germination of representative isolates. Therefore, the same volumes of acetone, methanol, and hydrochloric acid were added to PDA as a blank control. The serial dilutions of azoxystrobin were supplemented with 100 mg/liter of the alternative oxidase pathway inhibitor salicylhydroxamic acid (Huang et al. 2016).

Spore germination was used to determine the sensitivity of the spores to the fungicides. PDA containing different serial dilutions of the seven fungicides was prepared (Table 3), 200 μl of each of the fungicide-containing PDAs were applied to the slides, and 100 μl of conidia suspension (1×10^5 cfu/ml) was inoculated onto the slides after agar solidification, and they were incubated for 12 h at 25°C in the dark. Conidia germination was counted to determine the spore germination inhibition rate (Huang et al. 2016).

The mycelial growth rate method was used to determine the sensitivity of the mycelia to the fungicides. A disk of the representative isolates (diameter: 0.5 cm) was inoculated into the center of the fungicide-containing PDA, and a blank PDA medium was used as the control. After culturing for 5 days at 25°C , the colony diameters were measured using the cross lines method in two directions, and each treatment was replicated three times to calculate the inhibition rate (Hou et al. 2014; Xu et al. 2016).

$$\text{Inhibition rate (\%)} = \frac{(\text{Diameter of control fungal colony} - 0.5) - (\text{Diameter of treated fungal colony} - 0.5)}{\text{Diameter of control fungal colony} - 0.5} \times 100\%$$

The logarithm of the concentration of each treatment fungicide represented the independent variable (X), and the probability of the corresponding spore germination or mycelial growth inhibition rate represented the dependent variable (Y). The regression method was used to fit the virulence regression curve to yield the regression equation. In addition, the EC_{50} value was determined according to the regression equation (Hou et al. 2014; Xu et al. 2016).

Fig. 4. Effect of temperature on in vitro mycelial growth and conidia germination of *Ascochyta caulina*. Data were analyzed with SPSS statistics 19.0 by one-way ANOVA, and means were compared using Tukey's test at a significance level of $P = 0.05$. Different letters indicate significant differences ($P = 0.05$). Bars and error bars represent means $\pm$ SDs ($n = 3$).

Fig. 5. Germination of the conidia of *Ascochyta caulina* at different temperatures. **A**, Normal germination of the conidia at 0, 5, 10, 15, 20, and 25°C , respectively. **B**, Malformed germination of the conidia at 30 and 35°C .

Data statistics and analysis. One-way analysis of variance (ANOVA) was conducted using SPSS (version 19.0; IBM, Armonk, NY) and means were compared using Tukey's test at a significance level of $P = 0.05$. Homogeneity of the variance was tested before analysis.

Results

Field symptoms and loss survey. This disease primarily affected the stems of the quinoa plants. The lesions were diamond shaped and first appeared in the middle and lower parts of the stems, and gradually expanded upward. In the early stages of this disease, the lesions were seen to be pale or tan, and the margins between the lesions and healthy tissue were clear. There were slight depressions, as the plants were prone to drying and consequent shrinkage, and there were small round protrusions on the surface of the lesions (Fig. 1A). In the later stages, the lesions were black and accompanied by a large number of small black spots, the pycnidia of the pathogen, usually below the epidermis of the host. In severe cases, the lesions wrapped around the stem, causing lodging, foliar chlorosis, and leaf abscission. The diameters of lesions ranged from 5.9 to 9.6 cm (mean = 7.9 cm) (Fig. 1B–D). Quinoa black stem was found on both live and dead quinoa stems (Fig. 1B–E).

Morphology characteristics of the *Ascochyta* isolates. Thirty stem samples were selected for pathogen isolation, and a total of 13 isolates of pure fungi were obtained and stored. The 13 isolates were identical and showed typical colony characteristics and microscopic observations of *A. caulina* (teleomorph: *N. calvescens*). Hence, the representative isolates LMHS-3 and LMHS-5 were selected for the investigation from two different quinoa plantations in Jingle County, Shanxi Province. The colonies of the LMHS-3 and LMHS-5 isolates were semi-buried in PDA. The surface of the colony was velvety, centrally protruding, grayish white but yellowish brown at the margins, and feathery. The bottom of the colony was dark brown at the center and yellowish brown at the edges. After culturing on PDA for 7 days, the colony surface had fine particles that enlarged continuously and became black; there were maturing pycnidia for 20 days that were densely distributed in the center of the colony (Fig. 2A).

The hyphae were light brown with shrunken septa, with a width of 4.40 to 6.96 μm (mean = 5.56 μm). Adjacent hyphae were fused and entangled through frequent separation and branching of the multiple hyphae and were expanded around the center to form a primordial pycnidium. After the primordial pycnidium was formed, the hyphae continued to branch, enlarge, and produce brown pigments. In the center of the mycelial aggregation, part of the hyphae differentiated into thick-walled cells, the hyphae were closely entangled, and the spores gradually developed (Fig. 2B).

The pycnidia were observed to be hard textured, grayish-white or light brown in color, nearly spherical or pear-shaped, with a single chamber. The outer surfaces of the pycnidia had polygonal lattice patterns. The spore wall thickness ranged from 5.76 to 7.02 μm (mean = 6.52 μm). Pycnidia were 124.53 to 230.34 $\mu\text{m} \times 87.05$ to 227.27 μm in size (mean = 162.39 $\times$ 138.96 μm). The mycelia on the inner wall of the mature pycnidia were dense and smooth. Conidiogenous cells differentiated from the inner wall cells, were usually distributed in the cavity of the inner wall of the pycnidia, and were nearly conical in shape. The conidia grew individually and were elliptical or fusiform in shape, light brown in color, and usually had a septum, which was slightly constricted, erect or curved, oblong at the top, flat at the base, and 15.40 to 24.47 $\mu\text{m} \times 4.43$ to 7.70 μm in size (mean = 16.69 $\times$ 5.91 μm) (Fig. 2C).

Molecular characterization and phylogenetic analysis of the *Ascochyta* isolates. The LSU, SSU, and ITS gene sequences of isolates LMHS-3 and LMHS-5 were amplified, and the sequences obtained were submitted to GenBank (Table 2). A phylogenetic tree was constructed using *Phoma drobnjakensis* (CBS 269.92) as the outgroup. The results showed that the isolates LMHS-3 and LMHS-5 clustered in the same large branch with the three species in the *Neocamarosporium* Crous & Wingfield genus (*N. calvescens*, *N. obiones*, *N. chenopodii*) with a 100% bootstrap support rate. LMHS-3 and LMHS-5 clustered in a small branch with CBS 246.79 with a 100% bootstrap support rate, indicating that it had the closest relationship with *N. calvescens* (Fig. 3). Collectively, the morphology, molecular characterization, and pathology confirmed *N. calvescens* (anamorph: *A. caulina*) as the causal agent of quinoa black stem.

Effect of temperature on mycelial growth, conidia germination, and the pathogenicity of the *Ascochyta* isolates. The conidia of *A. caulina* (teleomorph: *N. calvescens*) germinated in a wide range of temperatures, from 0 to 35°C, and temperature had a significant effect on conidia germination (Fig. 4). The optimum temperature range for the germination of *A. caulina* conidia was 15 to 25°C; the germination rate ranged from 73 to 84%. Differences between the temperature treatments in this range were not significant. At temperatures below 15°C or above 25°C, the germination rates of the conidia were significantly decreased. At 10, 30, and 35°C, the germination rates were 55, 56, and 54%, respectively; the differences of which were not significant. At temperatures <5°C, the germination rate was reduced to less than 19% (Fig. 4).

Temperature had a significant effect on the mycelial growth of *A. caulina* (Fig. 4). The mycelia of *A. caulina* were found to grow best at 20 to 25°C, reaching colony diameters of 33.07 to 33.57 mm;

Fig. 6. Lesion length on quinoa stems that were inoculated with the *Ascochyta* isolate and incubated at different temperatures. Data were analyzed with SPSS statistics 19.0 by one-way ANOVA, and means were compared using Tukey's test at a significance level of $P = 0.05$. Different letters indicate significant differences ($P = 0.05$). Bars and error bars represent means $\pm$ SDs ($n = 3$).

differences between temperature treatments in this range were not significant. At temperatures below 20°C or above 25°C, the mycelial growth was significantly decreased. At temperatures below 5°C or above 35°C, the colony mean diameters were <20.00 mm (Fig. 4).

The conidia of *A. caulina* were able to germinate normally at temperatures in the range of 0 to 25°C. The germ tubes were able to germinate from the two ends or the sides of the conidia, from one or both ends, and rarely from the branches (Fig. 5A). At temperatures >30°C, the germination of *A. caulina* conidia was malformed, and presented

an altered mode of conidial germination whereby the germ tubes emerged from three or more sites on the conidia. In addition, the base or tip of the germ tubes were swollen or showed more branching (Fig. 5B).

Temperature had a significant effect on the pathogenicity of *A. caulina*. *A. caulina* was pathogenic between 15 and 25°C, and the length of the lesions ranged from 3.27 to 4.39 cm. The optimal pathogenic temperature of *A. caulina* was 25°C, and the length of the lesions was 4.39 cm, which was significantly higher than other treatments. When the temperature was below 10°C or above 35°C,

Fig. 7. Symptoms on *Chenopodium album*, *C. bryoniifolium*, and *C. quinoa* stems induced by inoculation of representative isolate of *Ascochyta caulina*. **A**, Pathogenicity of *A. caulina* on stems of *C. album*, *C. bryoniifolium*, and *C. quinoa* under climate chamber conditions. **B**, Pathogenicity of *A. caulina* on stems of *C. quinoa* at different days postinoculation (dpi) under greenhouse conditions. **C**, Symptoms induced on quinoa stem by inoculation of *A. caulina* under outdoor conditions. **D**, Pathogenicity of *A. caulina* on roots of *C. album* and *C. quinoa* under greenhouse condition, respectively.

the length of the lesions was significantly reduced to less than 0.50 cm (Fig. 6).

Pathogenicity tests of the *Ascochyta* isolates. Pathogenicity tests on the detached stems of *C. quinoa* showed typical symptoms 10 dpi, whereas no symptoms developed on those treated with sterile water (Fig. 7A). At 10 dpi, the inoculation sites of the detached stems of *C. quinoa* were densely covered with small pycnidia that were bulging and black in color (Fig. 7A). The two isolates did not cause any disease symptoms on the detached stems of *C. album* and *C. bryoniifolium* (Fig. 7A). The control plant stems of *C. album* and *C. bryoniifolium* remained healthy (Fig. 7A).

When the intact stems of *C. quinoa* were inoculated, typical symptoms were observed by 6 dpi. At 6 dpi, obvious lesions were found at

the inoculation sites, and the margins between lesions and healthy tissue were obvious, with a pale brown halo, a brown center, necrosis, and the accompanying small pycnidia (Fig. 7B). The lesions further developed and enlarged 10 dpi (Fig. 7B), similar to the symptoms observed in the field (Fig. 1B and C). There were no symptoms on the control (Fig. 7B). To fulfill Koch's postulates, the pathogenicity analysis was further inoculated on *C. quinoa* in outdoor conditions. At 15 dpi, typical symptoms appeared on the stems of *C. quinoa* in outdoor conditions (Fig. 7C), which were identical to the original ones that had been observed (Fig. 1C and D). The two isolates did not cause any disease symptoms on the roots of *C. quinoa* and *C. album* (Fig. 7D). The roots of the *C. quinoa* and *C. album* control plants remained healthy (Fig. 7D).

Fig. 8. Pathogenicity of *Ascochyta caulina* on *Chenopodium album*, *C. bryoniifolium*, and *C. quinoa*. **A**, Pathogenicity of *A. caulina* on detached leaves of *C. album*, *C. bryoniifolium*, and *C. quinoa* at 10, 15, 20, 25, 30, and 35°C, respectively. **B**, **C**, and **D**, Symptoms induced by inoculation of *A. caulina* on *C. album*, *C. bryoniifolium*, and *C. quinoa* under greenhouse conditions.

The optimum temperature for disease onset in the detached leaves of *C. album* ranged from 20 to 30°C. At 3 dpi, the lesions could be seen at the inoculation sites and the margins between lesions and healthy tissue were clear. At 6 dpi, the lesions had pale brown halos while their central parts were grayish white. The lesion sizes ranged from 0.62 to 2.53 cm at 8 dpi, when the small pycnidia were densely distributed at the inoculation sites in the 25°C treatment group. The optimum temperature for disease onset of the detached leaves of *C. bryoniifolium* ranged from 15 to 25°C. The lesions could be observed at the inoculation sites after 4 and 7 days as brown to light brown with small pycnidia attached. At 8 dpi, lesion sizes ranged from 2.32 to 3.46 cm, and the small pycnidia were most abundant at the sites inoculated at 25°C. The optimum temperature for disease onset on the detached leaves of *C. quinoa* ranged from 10 to 25°C. At 4 dpi,

lesions were visible at the inoculation sites and at 8 dpi the lesions were grayish white. Lesion sizes ranged from 0.37 to 2.16 cm on the red quinoa and from 0.24 to 1.87 cm on the white quinoa. At 15 to 25°C, the pycnidia were present at inoculation sites of the red quinoa leaves. The amount of small pycnidia on the red quinoa leaves was obviously more than that on the white quinoa leaves. During the experimental period, no disease occurred in the control groups or the 35°C groups of *C. album*, *C. bryoniifolium*, or *C. quinoa* leaves (Fig. 8A).

Pathogenicity analysis of *A. caulina* was further tested on intact leaves of *C. album*, *C. bryoniifolium*, and *C. quinoa* in the greenhouse. Typical brown spots clearly appeared on the intact leaves of *C. album*, *C. bryoniifolium*, and *C. quinoa* after 6, 7, and 8 dpi, respectively (Fig. 8B–D). Typical brown spots on the intact leaves

Fig. 9. Inhibition activities of seven fungicides on mycelial growth and conidial germination of *Ascochyta caulina*. Data were analyzed with SPSS statistics 19.0 by one-way ANOVA, and means were compared using Tukey's test at a significance level of $P = 0.05$. Different letters indicate significant differences ($P = 0.05$). Bars and error bars represent means $\pm$ SDs ($n = 3$).

Fig. 10. Morphology of conidia and germ tubes of *Ascochyta caulina* treated with different concentrations of seven fungicides for 12 h (A–G) and a control treatment (H). A, Carbendazim (1 μg/ml). B, Tebuconazole (0.0005 μg/ml). C, Prochloraz (0.5 μg/ml). D, Difenoconazole (0.0005 μg/ml). E, Thiophanate-methyl (0.5 μg/ml). F, Azoxystrobin (0.1 μg/ml). G, Mancozeb (0.1 μg/ml).

further developed, which were identical to the detached leaves of *C. album*, *C. bryoniifolium* and *C. quinoa* (Fig. 8).

The reobtained isolates from the inoculation-induced diseased stems and leaves showed that colony morphology and microscopic observations were identical to the ones for inoculation. Pathogenicity testing fulfilled Koch's postulates and indicated that the isolates LMHS-3 and LMHS-5 were pathogens.

Effects of seven fungicides on mycelia growth and conidia germination of the *Ascochyta* isolates. In order of decreasing toxicity on the conidial germination of *A. caulina*, the seven tested fungicides were mancozeb, azoxystrobin, thiophanate-methyl, difenoconazole, prochloraz, tebuconazole, and carbendazim; the EC₅₀ values were 5.426, 9.716, 62.114, 86.740, 104.946, 256.039, and 267.834 µg/ml, respectively. The fungicides that most strongly inhibited the conidial germination of *A. caulina* were mancozeb and azoxystrobin; the differences between the two were not significant, but the differences compared with the other five fungicides were significant (Fig. 9).

In order of decreasing toxicity on the mycelial growth of *A. caulina*, the seven tested fungicides were tebuconazole, difenoconazole, azoxystrobin, mancozeb, carbendazim, thiophanate-methyl, and prochloraz; the EC₅₀ values were 0.034, 0.047, 0.295, 0.303, 3.369, 4.957, and 6.534 µg/ml, respectively. The fungicides that most strongly inhibited the mycelial growth of *A. caulina* were tebuconazole and difenoconazole, followed by azoxystrobin and mancozeb (Fig. 9).

When cultured onto PDA containing either carbendazim, tebuconazole, or thiophanate-methyl, the conidial germination of *A. caulina* was slightly malformed, with slight swellings at the base or tip of the germ tube (Fig. 10A, B, and E). When cultured for 12 h on PDA containing either azoxystrobin or mancozeb, conidial germination was severely malformed, with an altered mode of conidial germination, whereby the germ tubes emerged from two or more sites on the conidia and swollen or more heavily branched bases or tips of the germ tubes (Fig. 10F and G). When cultured for 12 h on PDA containing the fungicides prochloraz or difenoconazole, the spores germinated normally (Fig. 10C and D). On PDA without any fungicide, the shape of the germ tubes was normal, branches rarely appeared, and the germ tubes emerged from both ends or from the central part of the conidia (Fig. 10H).

Discussion

In recent years, quinoa black stem has been common in quinoa planting areas in Jingle, Ningwu, Wutai, and Shanyin counties of

Shanxi Province, China. A survey among serious quinoa fields in Jingle County, Shanxi Province, revealed that in an environment of 15 to 25°C with an RH of 60 to 80%, quinoa black stem spread very rapidly, and resulted in lodging, leaf yellowing, and leaf shedding within 8 to 10 days. The disease usually appears on the stems at flowering stage and symptoms can progress up to the stage of maturity in Shanxi Province. The present study showed that both high temperatures and low temperatures affected the mycelial growth and conidial germination of *A. caulina* (Figs. 4 and 5), thereby affecting its pathogenicity (Fig. 6). This also could partly explain why quinoa black stem was more likely to develop under cooler conditions (15 to 25°C, RH = 55 ± 2%).

The teleomorph of *A. caulina* (teleomorph: *Neocamarosporium calvescens*) had not been found in the quinoa planting areas in Shanxi Province for three consecutive years, which might indicate that the disease hazard is attributed to anamorphs in those areas. It has been reported that *A. caulina* can cause leaf spots or stem lesions in eight species of *Atriplex* and eight species of *Chenopodium*, but not quinoa (Table 4) (Boerema et al. 1977, 1987; de Gruyter et al. 2009; van der Aa and van Kesteren 1979). Compared with the symptoms in other host species, *A. caulina* infected quinoa stems with relatively large lesions, being 5.9 to 9.6 cm in diameter in the Jingle quinoa plantations, which could cause lodging resulting in greater damage (Boerema et al. 1977). In comparison with the stem lesions caused by *Ascochyta hyalosporea*, the quinoa black stem lesions were more likely to develop under cooler conditions (15 to 25°C, RH = 55 ± 2%) (Li et al. 2017). *A. hyalosporea* is *N. chenopodii* and is closely related to *N. calvescens* (*A. caulina*) (de Gruyter et al. 2013). *A. hyalosporea* can infect quinoa seeds, seedling roots, and hypocotyls, stems, and leaves, resulting in leaf spots and stem lesions (Boerema et al. 1977, 1987; van der Aa and van Kesteren 1979). *A. caulina* isolates LMHS-3 and LMHS-5 mainly infected quinoa stems (Fig. 7B), but usually they do not infect the roots, seedlings of quinoa, or the stems of *C. album* and *C. bryoniifolium* (Fig. 7A and D). The leaves of *C. album* and *C. bryoniifolium* were easily infected and many pycnidia could be produced (Fig. 8). The inoculation revealed that *A. caulina* could infect the leaves of quinoa in the climate chambers and greenhouse (Fig. 8). However, the foliar symptoms observed on detached leaves and plants grown under greenhouse conditions were not typically observed in the Shanxi quinoa plantations. These results suggest that the infections of quinoa leaves might only develop under favorable conditions, and the quinoa leaf spots caused by *A. caulina* required further investigation.

Table 4. Strains isolated from black stem on quinoa in this study and reference strains of *Ascochyta* leaf spot or stem lesion on *Atriplex* spp. and other *Chenopodium* plants

Strain	Host	Substrate	Citation
-	<i>Atriplex hortense</i>	Leaf	van der Aa and van Kesteren 1979
-	<i>A. lacinata</i>		
-	<i>A. littoralis</i>		
CBS 246.79	<i>A. hastata</i>		
-	<i>A. nitens</i>	Living and dying leaf	
-	<i>A. patula</i>	Living leaf	
CBS 344.78	<i>A. hastata</i>	Stem	
CBS 343.78			
CBS 787.68			
-	<i>A. hortensis</i>		
-	<i>A. oblongifolium</i>	Dying stem	-
-	<i>Chenopodium album</i>	Leaf	van der Aa and van Kesteren 1979
-	<i>C. bonus-henricus</i>		
-	<i>C. glaucum</i>		
-	<i>C. hybridum</i>		
-	<i>C. murale</i>		
-	<i>C. viride</i>	Living leaf	
-	<i>C. suecicum</i>	Living or dying stem	
-	<i>C. vulvaria</i>	Stem	
LMHS-3	<i>C. quinoa</i>	Living stem	This study
LMHS-5			

Some species in the genus *Neocamarosporium* (Crous & Wingfield) are morphologically similar, which poses significant difficulties in the identification of pathogens (Crous et al. 2014; Grum-Grzhimaylo et al. 2016; Papizadeh et al. 2018; Wanasinghe et al. 2017). Historically, fungal taxonomists have used the morphological characteristics of pathogens on their hosts as an important basis for identification. In the present study, the morphology of *A. caulina* on quinoa was directly observed on sections, and it was found that the pycnidia, conidia, and conidiogenous cells of *A. caulina* that were infecting the quinoa were generally consistent with the *A. caulina* specimens described by Boerema et al. (1987), Ariyawansa et al. (2015), and Wanasinghe et al. (2017), among others. The conidia of the *A. caulina* isolates LMHS-3 and LMHS-5 that infected the quinoa were slightly shorter than those seen infecting other hosts, such as pigweed (*C. album*) and hastata orache (*Atriplex hastata*) (Boerema et al. 1987).

The model strain of *A. hyalospora* is CBS 206.80. In 2013, de Gruyter et al. systematically studied CBS 206.80 and CBS 246.79, and showed that there were significant differences in the morphology and molecular characteristics of the two isolates and that they were not the same species (de Gruyter et al. 2013). The morphology and molecular sequences of the isolates LMHS-3 and LMHS-5 were close to CBS 246.79 (Fig. 3). In 2013, Testen et al. reported that *Ascochyta* leaf spot of quinoa was caused by an *Ascochyta* sp. in the United States that was most closely related to CBS 432.77 (Testen et al. 2013). The model strain of *N. calvescens* (anamorph: *A. caulina*) was CBS 246.79. Because CBS 246.79 was similar to CBS 344.78, CBS 432.77, and CBS 523.66, its taxonomic status has been changed (Crous et al. 2014; de Gruyter et al. 2009; Grum-Grzhimaylo et al. 2016; Papizadeh et al. 2018; Wanasinghe et al. 2017). In 2009, de Gruyter et al. identified CBS 246.79 as *A. caulina* (*Pleospora calvescens*) through LSU and SSU sequence analysis and considered CBS 246.79 and CBS 344.78 to be the same species (de Gruyter et al. 2009). de Gruyter et al. (2013) used the ACT sequencing analysis to identify CBS 246.79, CBS 344.78, CBS 432.77, and CBS 523.66 as *P. calvescens*, *P. chenopodii*, *P. halimiones*, and *P. betae*, respectively, and distinguished between CBS 246.79 and CBS 344.78 (de Gruyter et al. 2013). In recent years, CBS 246.79, CBS 344.78, CBS 432.77, and CBS 523.66 have been reidentified as *N. calvescens*, *N. chenopodii*, *N. obiones*, and *N. betae*, respectively, based on the phylogenetics of multiple genes (e.g., LSU, ITS, SSU, rpb2, ACT) (Ariyawansa et al. 2015; Wanasinghe et al. 2017). The LSU sequences of LMHS-3 and LMHS-5 had 100% homology with those of *N. chenopodii* (CBS 344.78), *N. obiones* (CBS 432.77), and *N. betae* (CBS 523.66). Therefore, the LSU sequences cannot be used to distinguish *N. calvescens* from *N. obiones*, *N. chenopodii*, and *N. betae*. Based on the analysis of multiple genes such as LSU, SSU, and ITS, it was found that the LMHS-3 and LMHS-5 clustered in the same branch as *N. calvescens* from *Atriplex hastata* (CBS 246.79) with the 100% bootstrap support rate, and the relationship was closest to *N. calvescens* (Fig. 3). Collectively, the morphological, pathological, and molecular characterizations confirmed *N. calvescens* (anamorph: *A. caulina*) as the pathogen that causes quinoa black stem (LMHS-3 and LMHS-5). The hosts of the four isolates that have been reported, CBS 344.78, CBS 343.78, CBS 246.79, and CBS 787.68, are the leaves or stems of *A. hastata* (Table 4) (van der Aa and van Kesteren 1979). The present study found for the first time that isolates LMHS-3 and LMHS-5 can infect quinoa (Table 4).

When quinoa black stem incidence is severe, chemical control is essential (Huang et al. 2016). However, the toxicological effects of different types of fungicides differ, and even the toxicological effects of the same types of fungicide can differ among pathogens (Hou et al. 2014; Xu et al. 2016). For example, azoxystrobin has good inhibitory and teratogenic effects on conidia of *Alternaria suffruticosa* and *Cladosporium paenoniae*, and a good inhibitory effect on the mycelia of *Colletotrichum gloeosporioides*, but fewer inhibitory effects on the conidia (Hou et al. 2014; Xu et al. 2016). The present study showed that mancozeb and azoxystrobin had the strongest inhibitory effects on conidia germination in *A. caulina*, and that these two agents caused malformation of the conidia during germination. The results suggested that mancozeb and azoxystrobin might provide

preventive activities to protect quinoa from *A. caulina*. Tebuconazole and difenoconazole were shown to have the strongest inhibitory effects on the mycelial growth of *A. caulina* but had little effect on the conidial germination (Fig. 9). This could also partially explain how tebuconazole and difenoconazole might have curative activities to combat this disease.

In conclusion, a novel disease designated as quinoa black stem, which occurred in many Jingle quinoa cultivation regions of Shanxi, China, was characterized and distinguished from other known *Ascochyta* leaf spot diseases in this study. Using morphological characteristics and phylogenetic relationships, the causal agent was identified as *A. caulina* and a newly described pathogen of quinoa black stem. *A. caulina* isolates LMHS-3 and LMHS-5 were the first isolates that primarily infected quinoa stems and usually did not infect the roots and seedlings of quinoa. Our results can help improve the recognition, diagnosis, and management of this important disease. In the future, surveys of this pathogen are needed to assess its genetic diversity and to elucidate its areas of endemism, host range, infection mechanisms, and epidemiology, so that proactive measures can be taken to combat it.

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