

## 研究报告

## 一种由粉红粘帚霉引起的青稞根腐病

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**摘要:**【背景】根腐病在青稞生产中的危害日趋严重, 阻碍了青稞根腐病的有效防控及青海省青稞产业的发展。然而人们对青稞根腐病的研究甚少且病原菌不详。【目的】明确青稞根腐病发生的危害、病原及致病性, 为青稞根腐病的防控提供理论依据。【方法】采用常规的组织分离法分离青稞根腐病病原, 通过形态鉴定与分子鉴定结合的方法对病原进行鉴定, 并采用烧杯水琼脂法测定其致病性。【结果】共分离得到 4 株青稞根腐病病原菌, 鉴定为 *Clonostachys rosea*, 有较强的致病性且致病性差异显著, 经柯赫氏法则验证为青稞根腐病病原菌, 并且是一种新的青稞根腐病病原, 该类根腐病也是一种新的根腐类病害, 在国内外属首次发现。【结论】*Clonostachys rosea* 可引起青稞根腐病且致病性强。

**关键词:** 青稞; *Clonostachys rosea*; 根腐病; 致病性

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# *Clonostachys rosea*, a pathogen of root rot in naked barley (*Hordeum vulgare* L. var. *nudum* Hook. f.) on the Qinghai-Tibet Plateau, China

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**Abstract:** [Background] The increasingly serious root rot has posed a threat to the production of naked barley (*Hordeum vulgare* L. var. *nudum* Hook. f.) and hampered the prevention and control of the disease and the development of the naked barley industry in Qinghai province. However, the root rot of naked barley has been rarely studied and the pathogens are unclear. [Objective] This paper aims to clarify incidence and pathogens of the root rot in naked barley and the pathogenicity of the pathogens, which is expected to lay a theoretical basis for the prevention and control of the disease. [Methods] The conventional method of tissue isolation was adopted to isolate pathogens of root rot in naked barley. The pathogens were detected by both morphological identification and molecular identification, and their pathogenicity was determined with a beaker of water-agar. [Results] A total of 4 stains with strong and significantly different pathogenicity were isolated, which were identified as *Clonostachys rosea*. As verified by Koch's postulates, they were new pathogens of root rot of naked barley, and the induced root rot was found for the first time at home and abroad. [Conclusion] *C. rosea* can cause root rot in naked barley with strong pathogenicity.

**Keywords:** naked barley; *Clonostachys rosea*; root rot; pathogenicity

Naked barley (*Hordeum vulgare* L. var. *nudum* Hook. f.), a barley variant, is cultivated worldwide from Macedonia to Australia, Western Canada, and the Great Plains of the United States of America (USA). In China, naked barley is mainly distributed in the high-altitude areas of Tibet, Gansu, Qinghai, Yunnan, and Sichuan, where it is used both for grain and forage production<sup>[1]</sup>. Naked barley is rich in various amino acids, dietary fibers, vitamins,  $\beta$ -glucan and minerals, such as calcium, magnesium, phosphorus, zinc, manganese, and selenium. In addition, naked barley has unique effects on health, such as the reduction of blood lipids, enhancement of gastric motility, and prevention of altitude sickness and diabetes<sup>[2]</sup>.

Root rot has a major impact on barley

production, and it has been shown to reduce crop yield by as much as 10% in Canada and North Dakota, USA<sup>[3]</sup>. Compared to healthy barley, root-rotted barley exhibits severely reduced root and shoot fresh and dry weights as well as reduced grain number per plant and, ultimately, reduced yield<sup>[4]</sup>. Since the isolation of the pathogen *Fusarium culmorum* from the rhizome internodes of Albertan root-rotted barley, first by Mills<sup>[5]</sup> in 1972 and then by others<sup>[6-8]</sup>, other causal pathogens of barley root rot have been identified, including *Bipolaris sorokiniana*<sup>[3,6,9-13]</sup>, *Fusarium avenaceum*<sup>[6,14-15]</sup>, *Fusarium graminearum*<sup>[6,16]</sup>, *Fusarium poae*<sup>[17]</sup>, *Fusarium sporotrichioides*<sup>[18]</sup>, and *Rhizoctonia* spp.<sup>[4,19]</sup>.

We recently conducted a general survey to clarify the incidence and etiology of naked barley

root rot in Northwestern China. During our investigation in Qinghai, in 2016, we found root-rotted naked barley plants. These plants were collected and transported to the laboratory for the isolation and identification of the pathogen causing such symptoms. Following the morphological and molecular identification of the fungus, its pathogenicity was confirmed based on Koch's postulates. The results of the present study provide a reliable basis for research concerning the pathogenesis and control of naked barley root rot.

## 1 Materials and Methods

### 1.1 Disease survey and sampling

From June to August 2016, root rot of naked barley seedlings and adult plants was surveyed in Haiyan and Gangcha counties of Haibei prefecture, and Haidong city, Qinghai province, China.

### 1.2 Pathogen isolation and purification

Diseased roots were surface sterilized with 0.1% mercury bichloride solution for 10 s after washing with 70% ethanol for 2 to 3 s, and then rinsed four times with sterile water to remove chemical residues. The roots were then dried with sterile filter paper. The middle part of each root was sliced with a sterile scalpel, inoculated on potato dextrose agar (PDA), and incubated for 72 to 96 h at 25 °C. The two ends of growing hyphae observed in each root section after the incubation period were transferred to fresh PDA plates. The single spore separation method was used to purify the fungus, which was sub-cultured on potato sucrose agar (PSA) plates.

### 1.3 Morphological identification

The morphological characteristics of pure strains cultured on PSA medium were observed under a 40× optical microscope. The genus and species (if possible) were identified for each strain based on growth rate, spore morphology, and other microscopic characteristics as per identification keys<sup>[20]</sup>.

### 1.4 Molecular identification

The fungal strains were inoculated onto PDA medium, cultured at 25 °C for 5 days, and then

transferred onto potato dextrose broth and incubated in a shaker at 120 r/min and 25 °C for another 5 days. After this period, genomic DNAs were extracted from the mycelia using the Fungal DNA Kit (Omega Bio-Tek, USA) and used as templates for PCR amplification of the internal transcribed spacer region of ribosomal DNA (rDNA ITS region). The reaction system contained: 1.0 μL of template DNA, 2.5 μL of 10×buffer (containing 2.5 mmol/L Mg<sup>2+</sup>), 0.5 μL of *Taq* polymerase (5 U/μL), 1.0 μL of dNTPs (10 mmol/L), and 1 μL of each primer (±10 μmol/L), namely ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). This mixture was then diluted with double distilled water to 25 μL (total reaction volume) and mixed thoroughly within a 0.2 mL centrifuge tube. Any droplets on the tube wall were forced down to the tube bottom by centrifugation. PCRs were conducted using a thermal cycler under the following conditions: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 45 s, extension at 72 °C for 80 s; and final extension at 72 °C for 7 min. After PCR, 2 μL of each amplicon was electrophoresed on a 1% agarose gel to verify the amplified fragments, and those of correct size were sent to Tianyi Huiyuan Biotechnology Co., Ltd. Beijing (China), for sequencing on an ABI3730-XL sequencer (Applied Biosystems, USA). The sequences obtained were blasted to the national center for biotechnology information (NCBI) database and those corresponding to fungal strains were later submitted to the GenBank (NCBI) database. We constructed a phylogenetic tree using the neighbor-joining method in MEGA 7.0 to determine the taxonomic status of each identified strain.

### 1.5 Pathogenicity determination and strain re-isolation

'Zangqing 2000' was used as the test cultivar. After naked barely seeds were sprouted, uniform seeds were picked and placed in pots filled with irradiated flower soil and vermiculite (3:1). A 2.5 mm diameter piece of each fungal strain was

placed in 150 mL sterilized potato glucose liquid, cultivated 5 days at 25 °C, then poured into each pot (100 mL/pot) containing barley seedlings grown for 10 days. Uninoculated potato glucose liquid and sterile water were used as controls. Ten naked-barley plants were placed in each pot together with five replicates of each fungal strain. They were all cultivated at room temperature under natural light for 8 days, disease incidence was observed and graded as follows: Grade 0: Healthy and disease free, with green leaves and white roots and rhizomes; Grade 1: Black spots not exceeding 30% of the root area; Grade 2: Roots less than 50% blackened; Grade 3: Black spots exceeding 50% of the root area, stem base with yellow-brown patches, and stunted seedlings; Grade 4: Roots brittle and black spots exceeding 70% of the root area; stem base with yellow-brown patches, and seedlings dead or nearly dead.

Disease incidence (%) = Number of diseased seedlings / total number of seedlings × 100

Disease index (DI) (%) = (Σ Number of diseased plants in various grades × disease grading representative value) / (highest disease grade × total number of seeds) × 100.

Duncan's new multiple range test was used to analyze significant differences.

In accordance with Koch's postulates, we

randomly selected three diseased plants from each disease grade. The roots were gently cut, inoculated onto PDA medium, and then cultured at 25 °C for 7 days, followed by microscopic observation of spore morphology. During this process, colony morphology was observed continuously to confirm whether the strains were the same as those at the time of inoculation. The strains with a consistent morphology were identified as the root rot pathogens in naked barley.

## 2 Results and Analysis

### 2.1 Disease symptoms

Diseased plants were short and stunted, with yellow-brown patches at the stem base. The roots either had black spots or had become blackened and showed rotting; in severe cases, plants died (Figure 1).

### 2.2 Morphological identification

Four strains of pathogenic fungi were isolated and purified. In the early stages of growth, the colonies appeared white and soft; however, after 7 days of incubation, yellow pigments appeared on the surface of the colonies. Eventually, the color of the entire colonies changed to bright yellow and hyphae became thinner. The generator cells were monophialidic, transparent, and showed

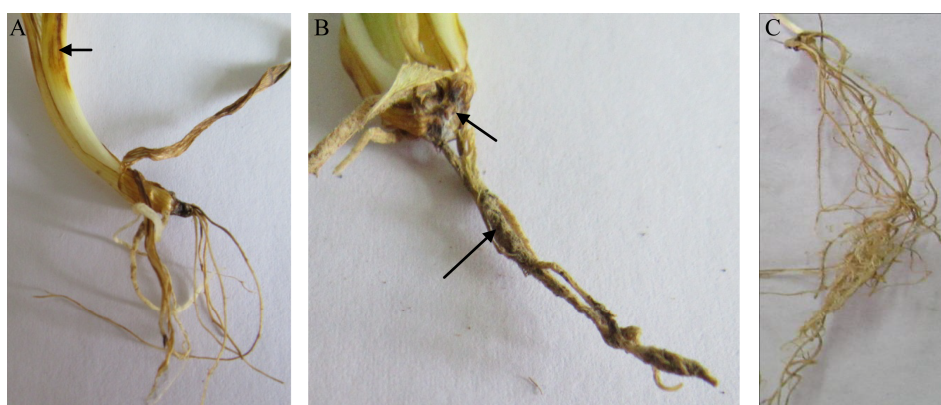


图1 青稞田间感染根腐病的症状 A: 茎基部有黄褐色病斑(箭头所指); B: 根部变黑腐烂(箭头所指); C: 健康的根

Figure 1 Root rot symptoms of naturally-infected naked barley. A: Yellow-brown patches at the stem base (where the arrow points); B: Blackened and rotten root showing black spots (where the arrow points); C: Healthy root.

verticillium-type branches. Numerous colorless microspores were produced. These microspores were oval, transparent, slightly bent, and  $(5.5\text{--}9.8)\ \mu\text{m} \times (1.8\text{--}3.5)\ \mu\text{m}$  in size (Figure 2), similar to those described by Ivabovó et al<sup>[20]</sup>. These strains were identified as *Clonostachys rosea* (Link) Schroers, based on their morphological characteristics.

### 2.3 Molecular identification

These sequences of the rDNA ITS region of the four isolated strains, i.e., NQ5-3, Q4-13, NQ4-5, and Q4-14 were submitted to GenBank under accession numbers KY365581, KY365587, KY365580, and KY365588, respectively. These sequences were 100% similar to *Clonostachys rosea* (Figure 3).

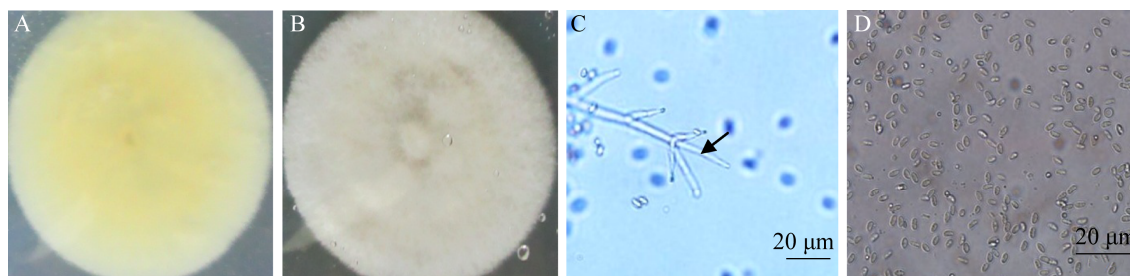


图2 *Clonostachys rosea* 的形态特征 A: 菌落背面; B: 菌落正面; C: 分生孢子梗(箭头所指); D: 分生孢子

Figure 2 Morphological characteristics of *Clonostachys rosea*. A: Colony reverse; B: Colony obverse; C: Conidiophore (where the arrow points); D: Conidia.

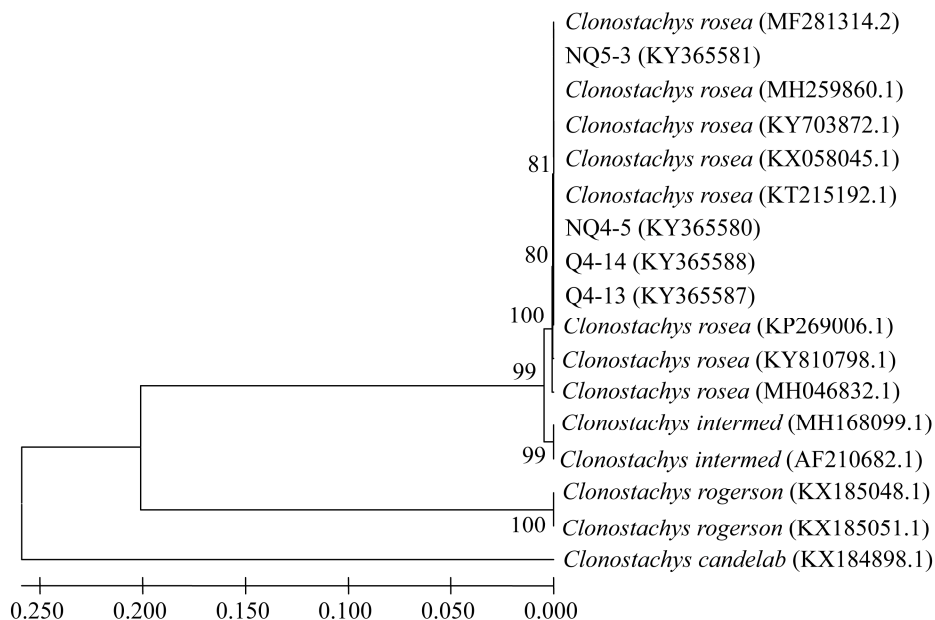


图3 利用平均距离法基于 rDNA ITS 序列构建的 *Clonostachys rosea* 的系统发育树 图中以大写字母开头的序列号为该菌株在 GenBank 中的登录号; 各分支的数字为 1 000 次重复计算的自展支持率, 图中自展支持率均大于等于 80; 标尺刻度为各菌株的遗传距离

Figure 3 Phylogenetic tree inferred from the dataset based on the rDNA ITS sequences of *Clonostachys rosea* using the UPGAM method. The serial number starting with capital letters in the figure are the entry number of the strain in GenBank. The number for each branch were calculated from 1 000 replications of the bootstrap values. RaxML bootstrap support values  $\geq 80$  are shown. The scale is the genetic distance of each strain.

## 2.4 Pathogenicity and re-isolation

*Clonostachys rosea* strains were significantly pathogenic (Table 1), and the symptoms exhibited by diseased plants were those evidenced in Figure 1. Control plants showed no signs of disease. The morphologies of the fungal strains were consistent with those of the strains re-isolated from all plants showing disease signs. These four strains were, therefore, confirmed to be the causal pathogens of naked barley root rot.

## 3 Discussion and Conclusion

*Clonostachys rosea* has been shown to be a typical biocontrol fungus with a widespread distribution that mainly inhibits pathogenic fungi, such as *Sclerotinia*, *Botrytis*, *Rhizoctonia*, and *Fusarium* species, among others<sup>[21]</sup>; it can control grey mold of fruits and vegetables<sup>[22]</sup>, barley mottle<sup>[23]</sup>, wheat scab<sup>[24]</sup>, corn stalk rot<sup>[25]</sup>, and potato black scurf<sup>[26]</sup>, as well as nematodes<sup>[27]</sup> and insects<sup>[28]</sup>. In the present study, all four isolated *C. rosea* strains were pathogenic and three of them were highly pathogenic with disease indexes >90. To our knowledge, this is the first report of this fungus as the cause of naked barley root rot. And other studies reported that *C. rosea* is the causal agent of broad bean crown rot and blight<sup>[29]</sup>. In addition, *C. rosea* is evidently pathogenic to tall

fescue<sup>[30]</sup>. However, *C. rosea* is a common endophytic fungus<sup>[31]</sup>. As a pathogen, whether the endophytic fungus show pathogenicity under the stress of environmental factors, namely whether *C. rosea* is an opportunistic pathogen remains to be further studied. In conclusion, the *C. rosea* species contains both pathogenic and beneficial strains, whose specific role may be related to the geographical location, environmental conditions, soil type and condition, crop species, and other factors. Therefore, further research is needed to verify whether the four *C. rosea* strains isolated here have biocontrol properties, and whether they are the cause or a precondition of disease in other crops.

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表1 *Clonostachys rosea* 对青稞的致病性

Table 1 Pathogenicity of *Clonostachys rosea* strains on naked barley

Strain	Root rot incidence (%)	Root rot index
Control	0	0
Q4-13	100±0a	95.83±2.89b
Q4-14	100±0a	92.50±2.50c
NQ4-5	100±0a	99.17±1.44a
NQ5-3	85±5b	21.67±2.89d

注: 表中数据为平均值±标准差。数据后的小写字母表示在  $P<0.05$  水平上的差异显著性, 不同字母则表示差异显著  
 Note: Data are means ± standard deviation. Means within columns followed by different lowercase letters are significantly different at  $P<0.05$ .

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## 征 稿 简 则

### 1 刊物简介与刊登内容

《微生物学通报》是由中国科学院微生物研究所和中国微生物学会主办, 以微生物学基础研究及技术创新与应用为主的综合性学术期刊。本刊为月刊, 为中文核心期刊、中国科技核心期刊、CSCD 核心期刊, 曾获国家优秀科技期刊三等奖, 中国科学院优秀科技期刊三等奖, 并在新闻出版署设立的“中国期刊方阵”中被列为“双效”期刊。从 2012 年至今, 本刊以国内“微生物学、病毒学类期刊”综合评价总分第一而蝉联“百种中国杰出学术期刊”称号, 而且入选 300 种“中国精品科技期刊”, 成为“中国精品科技期刊顶尖学术论文(F5000)”项目来源期刊。

本刊刊登内容包括: 工业、海洋、环境、基础、农业、食品、兽医、水生、药物、医学微生物学和微生物蛋白质组学、功能基因组、工程与药物等领域的最新研究成果、产业化新技术和新进展, 以及微生物学教学研究改革等。设置的栏目有: 研究报告、专论与综述、生物实验室、高校教改纵横、专栏等。

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3.1 来稿要求论点明确, 数据可靠, 简明通顺, 重点突出。

3.2 英文摘要写作注意事项: (1) 建议使用第一人称, 以此可区分研究结果是引用文献还是作者所得; (2) 建议用主动语态, 被动语态表达拖拉模糊, 尽量不用, 这样可以避免长句, 以求简单清晰; (3) 建议使用过去时态, 要求语法正确, 句子通顺; (4) 英文摘要的内容应与中文摘要一致, 但可比中文摘要更详尽, 写完后务必请英文较好且专业知识强的专家审阅定稿后再投稿; (5) 摘要中不要使用缩写语, 除非是人人皆知的, 如: DNA、ATP 等; (6) 在英文摘要中不要使用中文字体标点符号。

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(下转 p.644)



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## 征 稿 简 则

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- [1] Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model[J]. *Cell*, 1999, 96(1): 47-56
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