

灰葡萄孢菌引起的向日葵叶斑和盘腐及热胁迫对病菌菌核形成的影响

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摘要:【背景】2022年8–9月, 从甘肃省兰州市种植的食用向日葵田中采集到零星发病的叶斑和盘腐病害标样。【目的】明确叶斑病和盘腐病的病原及其生物学特性。【方法】采用单孢分离法进行病原菌分离; 通过 Koch's 法则明确分出病菌的致病性; 采用形态学和分子生物学方法对病原菌进行种类鉴定; 通过平板法测定试验菌株的适宜生长温度; 观察经 30 °C (生长抑制温度)培养后试验菌株产菌核特性的变化。【结果】分离得到 12 株菌落形态不同的葡萄孢属菌株。试验菌株 SKY-A–SKY-D 接种食用向日葵离体叶片和花盘可引起与自然发病相似的症状, 接种发病的叶片和花盘病组织上原接种菌的分出率达 100%。试验菌株在 PDA 平板上 15 °C 和 20 °C 培养 10–40 d, 大型分生孢子无色至淡褐色, 单胞, 卵圆形、长椭圆形、球形、近球形、棒状或不规则形, $[6.0-14.2 (-20.1)] \mu\text{m} \times [6.0-10.4 (-14.9)] \mu\text{m}$; 菌核表生, 黑色, 球形、近球形、椭圆形或不规则形, $(0.5-11.1) \text{mm} \times (0.5-5.0) \text{mm}$; I 型微菌核红褐色至黑褐色, 表生或埋生, $(26.9-492.5) \mu\text{m} \times (14.9-149.3) \mu\text{m}$, 微菌核的组成细胞大小为 $(12.7-35.0) \mu\text{m} \times (11.9-25.3) \mu\text{m}$; II 型微菌核近黑色, 埋生, $(35.8-373.1) \mu\text{m} \times (23.9-229.9) \mu\text{m}$, 微菌核的组成细胞大小为 $(8.2-16.4) \mu\text{m} \times (8.2-14.9) \mu\text{m}$; 假微菌核由特化的附着胞构成, 暗褐色至近黑色, 埋生, $(32.0-447.8) \mu\text{m} \times (19.4-358.2) \mu\text{m}$ 。试验菌株的菌丝适宜生长温度为 20–25 °C; 菌核产生温度为 5–25 °C。30 °C 培养 7 d 后转至 20 °C 培养 14 d, 可诱导 2 个不产菌核的菌株 SKY-B 和 SKY-C

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产生菌核, 突变株在继代培养时保持其产菌核能力。BLASTn 分析结果显示, 试验菌株 SKY-A–SKY-D 的 rDNA-ITS 序列(国家微生物科学数据中心登录号为 NMDCN00038NP–NMDCN00038NS)与已知灰葡萄孢菌(*Botrytis cinerea*)序列的相似性达 99.8%–100.0%。g3pdh、hsp60 和 rpb2 多基因系统发育分析结果显示, 4 个试验菌株被聚在 *B. cinerea* 的不同亚群里。【结论】引起向日葵叶斑和盘腐的病原菌被鉴定为 *B. cinerea*, 这是灰葡萄孢菌引起向日葵叶斑病和盘腐病在我国西北地区的首次报道。首次发现灰葡萄孢菌可以产生 2 种类型的微菌核。30 °C 热胁迫处理可诱导不产菌核的灰葡萄孢菌产生菌核, 突变株的产菌核能力可遗传。

关键词: 向日葵; 叶斑; 盘腐; 灰葡萄孢菌; 菌核; 微菌核; 假微菌核; 热胁迫

Leaf blotch and head rot caused by *Botrytis cinerea* on sunflower and effect of heat stress on sclerotium formation of the pathogen

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Abstract: [Background] In August and September 2022, the plant samples of leaf blotch and head rot were collected from sporadic diseased fields of confectionery sunflower (*Helianthus annuus*) in Lanzhou, Gansu, China. **[Objective]** To identify and characterize the pathogens of leaf blotch and head rot. **[Methods]** We isolated the pathogens by the single spore isolation method, measured the pathogenicity of the isolates based on Koch's postulates, and identified the isolates by morphological observation and molecular biological methods. Furthermore, we determined the optimum growth temperatures of the isolates by the plate culture method, and the changes of sclerotium formation characteristics of the tested isolates were observed after cultured at 30 °C (growth inhibition temperature). **[Results]** Twelve *Botrytis* isolates with different colony morphology were isolated from the diseased plant samples. The inoculation of four isolates, SKY-A to SKY-D, on detached sunflower leaves and heads induced the symptoms similar to those of natural diseases in the field, and the re-isolation rates of inoculated isolates from the infected leaves and heads were 100%. When the four isolates were cultured on PDA plates at 15 °C and 20 °C for 10–40 d, macroconidia were pale to light brown, unicellular, oval, oblong, spherical, subspherical, clavate or irregular shaped, (6.0–14.2 (–20.1)) μm×(6.0–10.4 (–14.9)) μm. The sclerotia were superficial, black, spherical, subspherical, ellipse or irregularly shaped,

(0.5–11.1) mm×(0.5–5.0) mm. Type-I microsclerotia were reddish brown to dark brown, superficial or submerged, (26.9–492.5) μm ×(14.9–149.3) μm , with the cell sizes of (12.7–35.0) μm ×(11.9–25.3) μm . Type-II microsclerotia were nearly black, submerged, (35.8–373.1) μm ×(23.9–229.9) μm , with the cell sizes of (8.2–16.4) μm ×(8.2–14.9) μm . Pseudo-microsclerotia consisting of specialized appressoria were dark brown to nearly black, submerged, (32.0–447.8) μm ×(19.4–358.2) μm . The optimal temperature range for mycelial growth and the temperature range for sclerotium formation were 20–25 °C and 5–25 °C, respectively. After incubation at 30 °C for 7 d and then at 20 °C for 14 d, SKY-B and SKY-C originally incapable of forming sclerotia formed sclerotia. Moreover, the mutants retained the ability of forming sclerotia in subculturing. BLASTn analysis showed that the rDNA-ITS sequences of SKY-A to SKY-D (National Microbiology Data Center Acc. No. NMDCN00038NP–NMDCN00038NS) had the similarity of 99.8%–100.0% with those of *Botrytis cinerea* strains in GenBank. The phylogenetic analysis based on the glyceraldehyde 3-phosphate dehydrogenase gene (*g3pdh*), heat shock protein 60 gene (*hsp60*), and DNA-dependent RNA polymerase subunit II gene (*rpb2*) showed that the four isolates were clustered in different subgroups of *B. cinerea*. **[Conclusion]** The pathogens causing leaf blotch and head rot on confectionery sunflower were identified as *B. cinerea*. This is the first report of leaf blotch and head rot caused by *B. cinerea* on confectionery sunflower in the Northwest China. For the first time, we discover that *B. cinerea* can produce two types of microsclerotia. The heat stress at 30 °C induced the *B. cinerea* isolates incapable of forming sclerotia to form sclerotia, and the sclerotium-forming ability of the mutants could be inherited.

Keywords: sunflower (*Helianthus annuus*); leaf blotch; head rot; *Botrytis cinerea*; sclerotium; microsclerotium; pseudo-microsclerotium; heat stress

向日葵(*Helianthus annuus* L.)为菊科向日葵属一年生草本双子叶植物,是世界四大油料作物之一,具有生育期短、耐贫瘠、耐盐碱、抗干旱、适应性强及易管理等特点,在世界范围内被广泛种植。我国向日葵常年种植面积约 106 万 hm^2 ,年总产量约 285 万 t,居世界第 4 位^[1]。我国种植的向日葵以食用向日葵为主,食用向日葵面积占向日葵总面积的 95.0% 以上^[2]。向日葵作为我国北方地区重要的油料经济作物,主要分布在内蒙古、新疆、吉林、辽宁、黑龙江、山西、陕西、河北、甘肃、宁夏等黄河以北地区,10 个优势主产省份的向日葵籽产量占全国向日葵籽总产量的 97.5%^[3]。

灰葡萄孢菌(*Botrytis cinerea* Pers.)是一种

重要的坏死性植物病原菌,有超过 200 种作物因灰霉病而遭受严重损失,蔬菜(卷心菜、生菜、花椰菜、豆类等)和小型水果(葡萄、草莓、覆盆子、黑莓等)是受害最严重的作物,一些重要的大田作物也因灰霉病而遭受严重损失,向日葵是受害严重的重要油料作物^[4]。

由于 *B. cinerea* 的寄生性和腐生性都很强(寄生的占 33%,腐生的占 67%)^[5],虽然在吉林和内蒙古的向日葵上有 *B. cinerea* 的寄主记录^[5],但作为向日葵病害,除台湾省外^[6],我国其他地区未见报道。

2022 年 8–9 月,从甘肃省兰州市种植的食用向日葵田中采集到零星发病的叶斑和盘腐病害标样,在病样上可见到分枝的分生孢子梗和

葡萄状着生的单细胞分生孢子。本研究旨在明确该病害的病原及其生物学特性,为病害的科学防治提供依据。

1 材料与方法

1.1 病害标样采集

2022年8月19日,从甘肃省兰州市永登县中川镇元山村采集1份产生不规则褐色叶斑症状的向日葵叶片(样本1);2022年9月27日,从甘肃省兰州市兰州新区秦川镇六墩村采集2份生有褐色病斑的向日葵花盘(样本2和样本3)。将样本带回实验室,用于病害症状观察、描述和病原菌分离。

1.2 培养基

PDA和PDB, BD公司。

1.3 主要试剂和仪器

植物DNA提取试剂盒(通用型)、PCR引物、DL5000 DNA Marker、TSINGKE I5 Mix和DNA凝胶回收试剂盒,擎科生物科技有限公司。

离心机、PCR仪和测序仪,赛默飞世尔科技公司;电泳仪、电泳槽和凝胶成像仪,北京君意东方电泳设备有限公司;水浴锅,北京中兴伟业仪器有限公司;板式离心机,湖南湘仪实验室仪器开发有限公司。

1.4 病原菌分离

用无菌接种针挑取少许产生于病害标样上的病菌分生孢子移入50 mL无菌水中,蘸取少许孢子悬浮液在PDA平板上划线培养,20–25 °C培养1–2 d,挑取单孢子菌落20–25 °C继续纯化培养1–2 d,纯化后的菌株接种于PDA斜面保存。

1.5 致病性测定

叶片接种:试验菌株在PDA平板20–25 °C培养4–6 d,取直径5 mm菌丝块接种于向日葵离体叶片上(叶片取自约50 d苗龄的盆栽向日

葵植株,自来水冲洗干净),每叶片接种1枚菌丝块,以接种相同大小的无菌PDA培养基块为对照。每处理10个叶片,置于放有湿滤纸的直径20 cm培养皿内,18–22 °C、自然散射光下保湿培养。

花盘接种:试验菌株在PDA平板20–25 °C培养4–6 d,取直径5 mm菌丝块接种于向日葵花盘背面(花托)(花盘取自大田,用75%乙醇擦拭花盘背面进行表面消毒),每花盘接种2–4枚菌丝块,以接种相同大小的无菌PDA培养基块为对照,每处理11个花盘,置于18 cm×12 cm×7 cm塑料保鲜盒或30 cm×30 cm塑料袋中,18–22 °C、自然散射光下保湿培养。接种4 d后移除接种的菌丝块和无菌PDA培养基块。

1.6 病原菌适宜生长温度测定及形态特征观察

试验菌株在PDA平板上20 °C黑暗培养4 d后,取直径5 mm菌丝块接种于PDA平板中央,置不同温度(5、10、15、20、25、30和35 °C)下恒温黑暗培养,重复3皿。培养2 d时用十字交叉法测量菌落直径,持续培养至21 d,观察不同温度条件下菌落形态及菌核产生情况。

试验菌株在PDA平板上20 °C和15 °C培养10–40 d,观察、测量分生孢子梗、分生孢子、菌核和微菌核的形态和大小,每个目标的测量≥30个数值。依据病菌培养特征和显微形态特征进行种类鉴定。

1.7 热胁迫对病菌产生菌核的影响

试验菌株在PDA平板上30 °C黑暗培养7 d后,将培养皿转放至20 °C下恒温黑暗条件下继续培养,观察试验菌株恢复生长及菌核产生情况,重复3皿。以热胁迫后的诱变菌株为接种体,取直径5 mm菌丝块接种于PDA平板中央,置20 °C和15 °C黑暗培养21 d,观察诱变菌株变异性状的可遗传性,重复3皿。

1.8 病原菌分子生物学鉴定

1.8.1 DNA 提取及测序

DNA 提取及测序工作委托擎科生物科技有限公司完成。

DNA 提取：取在 PDB 中 20–25 °C 培养 7 d 的试验菌株的菌丝，使用 TSINGKE 植物 DNA 提取试剂盒(通用型)按使用说明提取总 DNA。

目的基因 PCR 扩增及测序：PCR 反应体系(30 μL)：TSINGKE I5 Mix 15.0 μL，正、反向引物(10 μmol/L)各 1 μL，DNA 模板 1 μL，ddH₂O 12.0 μL。引物序列及退火温度见表 1。PCR 反应条件：98 °C 3 min；98 °C 10 s，55 °C/64 °C 15 s，72 °C 15 s，37 个循环；72 °C 5 min；4 °C 保存。PCR 产物用 DNA 凝胶回收试剂盒进行切胶回收、Sanger 法测序。

测序结果在 NCBI 数据库(<http://www.ncbi.nlm.nih.gov>)进行 BLASTn 分析，并提交国家微生物科学数据中心(National Microbiology Data Center, NMDC)获得登录号。

1.8.2 构建系统发育树

基于试验菌株的 *g3pdh*、*hsp60* 和 *rpb2* 基

因序列构建系统发育树：从 GenBank 核酸序列数据库中下载有可靠文献支撑的相关菌株的 *g3pdh*、*hsp60* 和 *rpb2* 基因序列(表 2)，以核盘菌(*Sclerotinia sclerotiorum* (Lib.) de Bary)为外群，用 ClustalX 1.83 软件对目的序列进行比对(alignment)，比对后的序列用 MEGA 7.0 软件进行 *g3pdh+hsp60+rpb2* 序列拼接，以最大似然(maximum likelihood, ML)法和最大简约(maximum parsimony, MP)法构建系统发育树^[17]，1 000 次 bootstrap 检验计算系统树中节点的置信度。

2 结果与分析

2.1 病害症状

向日葵叶片上的病斑为不规则形溃烂状干斑，黄褐色至灰黑色，具黄绿色晕纹，病斑大小约 5.0 cm×2.2 cm；病叶在 20–25 °C 保湿培养 1 d，病斑上长出灰白色菌丝(叶片背面的菌丝较正面丰盛)。向日葵花盘上的病斑黄褐色，病斑大小约 7.0 cm×4.5 cm，表面可见极薄或稍厚的灰色霉层；切开病斑，灰色菌丝长满相连的腔室，被侵染的海绵组织呈褐色，并已抵近向日葵瘦果(图 1)。

表 1 本研究所用引物序列

Table 1 Primer sequences used in this research

目标区域	引物名称	引物序列	退火温度	参考文献
Target region	Primer name	Primer sequence (5'→3')	Annealing temperature (°C)	Reference
rDNA-ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	55	[7]
	ITS4	TCCTCCGCTTATTGATATGC		
	ITS1	TCCGTAGGTGAACCTGCGG	55	
	ITS4	TCCTCCGCTTATTGATATGC		
<i>g3pdh</i>	G3PDH-F	ATTGACATCGTCGCTGTCAACGA	64	[8]
	G3PDH-R	ACCCCACTCGTTGTCGTACCA		
<i>hsp60</i>	HSP60-F	CAACAATTGAGATTTGCCACAAG	55	[8]
	HSP60-R	GATGGATCCAGTGGTACCGAGCAT		
<i>rpb2</i>	RPB2-F	GATGATCGTGATCATTTCGG	55	[8]
	RPB2-R	CCCATAGCTTGCTTACCCAT		

rDNA-ITS：细胞核核糖体 DNA，内转录间隔区；*g3pdh*：3-磷酸甘油醛脱氢酶基因；*hsp60*：热激蛋白 60 基因；*rpb2*：DNA 依赖性 RNA 聚合酶亚基 II 基因

rDNA-ITS: Nuclear rDNA, internal transcribed spacer region; *g3pdh*: Glyceraldehyde-3-phosphate dehydrogenase; *hsp60*: Heat-shock protein 60; *rpb2*: DNA-dependent RNA polymerase subunit II.

表 2 从 GenBank 下载的 *Botrytis cinerea* 及相关种类的蛋白质编码基因序列信息Table 2 The protein-coding gene sequences of *Botrytis cinerea* and related species downloaded from GenBank and their information

种属 Species	菌株编号 Strain No.	寄主 Host	地理来源 Locality	蛋白质编码基因登录号 Protein-coding gene accession number			参考文献 Reference
				<i>g3pdh</i>	<i>hsp60</i>	<i>rpb2</i>	
<i>Botrytis cinerea</i>	COAD 2805 (=B74)	<i>Dahlia</i> sp.	Brazil: Minas Gerais	MN547187.1	MN561097.1	MN561153.1	[9]
	COAD 2824 (=B75)	<i>Helianthus annuus</i>	Brazil: Minas Gerais	MN547188.1	MN561098.1	MN561154.1	[9]
	LSR-1	Lettuce	China	ON911590.1	ON911589.1	—	[10]
	MUCL87*	—	Netherlands	AJ705004.1	AJ716065.1	AJ745676.1	[8]
	Poly-4	<i>Fagopyrum tataricum</i>	China: Gansu	MG846499.1	MG846504.1	MG846509.1	[11]
	Poly-5	<i>Fagopyrum tataricum</i>	China: Gansu	MG846500.1	MG846505.1	MG846510.1	[11]
	SAS405	—	Italy	AJ705005.1	AJ716066.1	AJ745678.1	[8]
<i>Botrytis eucalypti</i>	CERC7170*	<i>Eucalyptus urophylla</i> × <i>Eucalyptus grandis</i>	China: Guangdong	KX301020.1	KX301024.1	KX301028.1	[12]
	CERC7208	<i>Eucalyptus urophylla</i> × <i>Eucalyptus grandis</i>	China: Guangdong	KX301021.1	KX301025.1	KX301029.1	[12]
<i>Botrytis fabae</i>	CBS 109.57	—	Netherlands	AJ705013.1	AJ716074.1	AJ745685.1	[8]
	MUCL98*	—	Spain	AJ705014.1	AJ716075.1	AJ745686.1	[8]
<i>Botrytis macadamiae</i>	BRIP 72276a	<i>Macadamia integrifolia</i>	Australia	MZ344225.1	MZ344236.1	MZ356232.1	[13]
	BRIP 72295a*	<i>Macadamia integrifolia</i>	Australia	MZ344226.1	MZ344237.1	MZ356233.1	[13]
<i>Botrytis pelargonii</i>	CBS497.50*	—	Norway	AJ704990.1	AJ716046.1	AJ745662.1	[8]
	MUCL1152	—	Norway	AJ705029.1	AJ716090.1	AJ745701.1	[8]
<i>Botrytis polyphyllae</i>	YN1	<i>Paris polyphylla</i>	China: Yunnan	MG387957.1	MG431978.1	MG431975.1	[14]
	YN3*	<i>Paris polyphylla</i>	China: Yunnan	MG387959.1	MG431980.1	MG431977.1	[14]
<i>Botrytis pseudocinerea</i>	10091	<i>Vitis vinifera</i>	France: Versailles	JN692414.1	JN692400.1	JN692428.1	[15]
	X1303	Blueberry	USA: California	KJ796654.1	KJ796658.1	KJ796650.1	[16]
<i>Sclerotinia sclerotiorum</i>	484	—	—	AJ705044.1	AJ716048.1	AJ745716.1	[8]

—: 不详; *: 模式、新模式或附加模式菌株

—: Unknown; *: Type, neotype or epitype strain.

2.2 病原菌分离结果

从 3 份病害标样上共分离得到 12 株葡萄孢菌。选择其中 4 株(SKY-A-SKY-D)为代表性菌株进行后续的研究工作。

2.3 致病性测定结果

接种 SKY-A-SKY-D 后 15 h, 向日葵叶片开始发病显症, 接种后 2 d, 病叶率达 100%; 接种后 42 h, 向日葵花盘开始发病显症, 接种

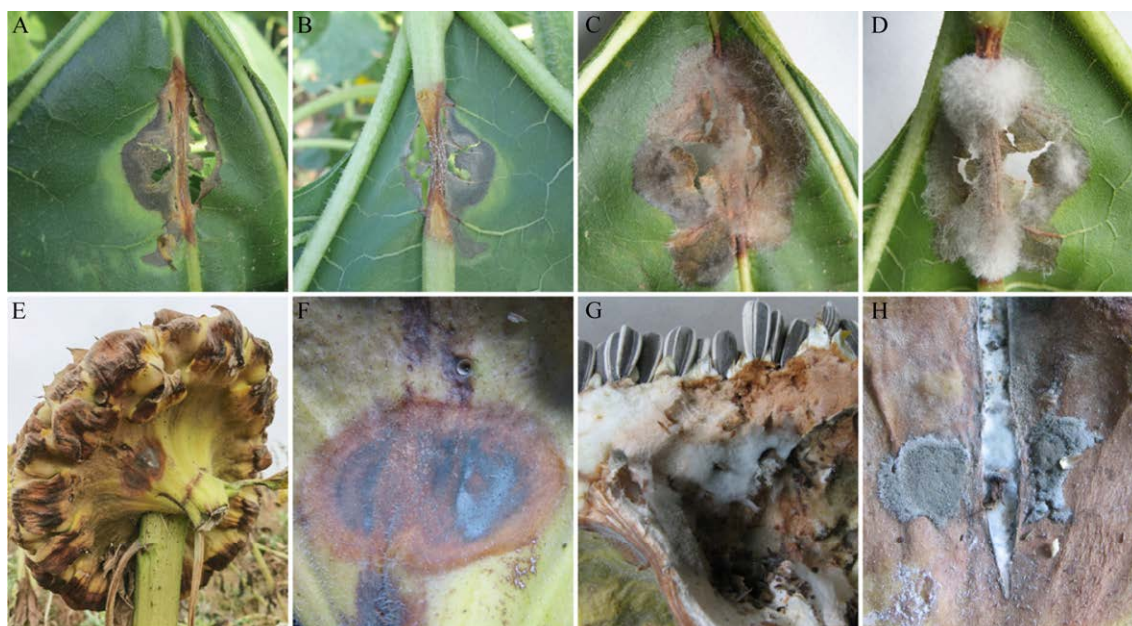


图 1 灰葡萄孢菌引起的向日葵叶斑病和盘腐病症状 A–D: 样本 1, 病叶症状(A、B: 田间症状; C、D: 病叶在湿滤纸上 20–25 °C 保湿培养 1 d; A、C: 叶片正面; B、D: 叶片背面). E–G: 样本 2 (E: 正在田间晾晒的罹病花盘; F: 花托上的病斑(花盘采集后在 25 °C 放置 4 h); G: 病盘剖面). H: 样本 3, 示罹病花盘病斑上的灰色菌丝和产孢层

Figure 1 Symptoms of leaf blotch and head rot caused by *Botrytis cinerea* on sunflower. A–D: Sample No. 1, symptoms of leaf blotch. A and B: In field. C and D: Incubated on moistened filter paper for 1 d at 20–25 °C. A and C: The obverse side. B and D: The reverse side. E–G: Sample No. 2. E: Diseased flower tray was drying in the field. F: Lesions on receptacle (sample was collected and placed in room for 4 h at 25 °C). G: Vertical section of the diseased head. H: Sample No. 3, showed that grey mycelia and conidial masses on lesion of diseased head.

后 6 d, 病盘率达 81.8%–100.0%。对照未发病。接种发病症状与自然发病症状相似, 发病花盘和叶片原接种菌的分出率达 100% (表 3, 图 2)。

2.4 病原菌适宜生长温度及形态特征

试验菌株的菌丝适宜生长温度为 20–25 °C (图 3)。菌株 SKY-A 产生菌核的温度为 5–20 °C, 菌株 SKY-D 产生菌核的温度为 5–25 °C。

在 PDA 平板上 20 °C 培养 14 d, 菌株 SKY-A 和 SKY-D 气生菌丝稀疏, 产生大量菌核, 菌核表生, 黑色, 球形、近球形、椭圆形或不规则形, 菌核大小为(0.5–11.1) mm×(0.5–5.0) mm, 菌核产

生的位置、菌核大小和数量在菌株和重复间存在较大差异; 菌株 SKY-B 和 SKY-C 气生菌丝丰茂, 产生大量聚生的灰色菌丝球, 不产生菌核(图 4)。

试验菌株在 PDA 平板上 20 °C 黑暗培养 10 d, 分生孢子梗长度可超过 1 cm; 分生孢子单细胞, 近无色至淡褐色, 卵圆形、球形或近球形, 基部平截、钝圆或尖锐, (6.0–14.2) μm×(6.0–10.4) μm; 分生孢子萌发可产生 1–4 个芽管。在 PDA 平板上 15 °C 黑暗培养 21 d, 球形孢子的直径可达 14.9 μm; 还观察到棒状和不规则形分生孢子, 棒状孢子的长度可达 20.1 μm (图 5)。

表 3 试验菌株对向日葵叶片和花盘的致病性

Table 3 Pathogenicity of four *Botrytis cinerea* isolates for sunflower leaf and head

菌株 Isolate	接种部位 Inoculation position	接种叶片数 或花盘数 Number of inoculation leaf or head	接种点数 Number of inoculation site	发病叶片数 或花盘数 Number of infection leaf or head	病叶率或病 盘率 Rate of infection leaf or head (%)	发病点数 Number of infection site	病点率 Rate of infection site (%)	病斑大小 Lesion size (mm)
SKY-A	Leaf	10	10	10	100	10	100	(35.0–57.0)×(30.0–38.0) (45.7±7.7)×(34.9±3.0)*
	Head	11	31	11	100	30	96.8	(5.0–55.0)×(5.0–55.0) (15.2±9.2)×(12.5±8.3)*
SKY-B	Leaf	10	10	10	100	10	100	(42.0–58.0)×(35.0–45.0) (51.2±5.8)×(39.6±3.6)*
	Head	11	34	11	100	24	70.6	(6.0–30.0)×(5.0–28.0) (13.2±7.0)×(11.8±6.7)*
SKY-C	Leaf	10	10	10	100	10	100	(34.0–58.0)×(30.0–40.0) (41.5±7.0)×(34.4±3.1)*
	Head	11	33	9	81.8	25	75.8	(9.0–25.0)×(5.0–21.0) (14.8±4.8)×(11.8±4.1)*
SKY-D	Leaf	10	10	10	100	10	100	(35.0–60.0)×(28.0–41.0) (49.0±8.7)×(34.5±4.1)*
	Head	11	33	9	81.8	27	81.8	(5.0–32.0)×(5.0–26.0) (17.6±7.4)×(14.4±6.9)*

调查时间：花盘接种后 6 d 调查；叶片接种后 3 d 调查。病斑大小以范围表示。*：平均数±标准差。试验期间的温度：18–22 °C

Survey time: 6 days post inoculation for heads; 3 days post inoculation for leaves. Lesion sizes are expressed as scope of length and width. *: the mean ± standard deviation. Temperature during test: 18–22 °C.

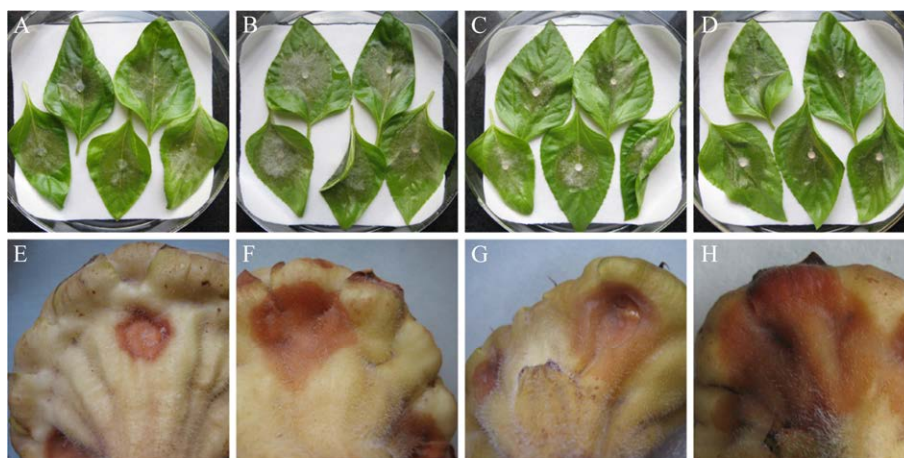


图 2 向日葵叶片和花盘接种发病症状 A–D：叶片接种后 3 d；E–H：花盘接种后 6 d。A、E：接种菌株 SKY-A；B、F：接种菌株 SKY-B；C、G：接种菌株 SKY-C；D、H：接种菌株 SKY-D。试验期间的温度：18–22 °C

Figure 2 Symptoms of artificial inoculation on sunflower leaves and heads. A–D: On leaves, for 3 d after inoculation. E–H: On heads, for 6 d after inoculation. A and E: Isolate SKY-A inoculated. B and F: Isolate SKY-B inoculated. C and G: Isolate SKY-C inoculated. D and H: Isolate SKY-D inoculated. Temperature during test: 18–22 °C.

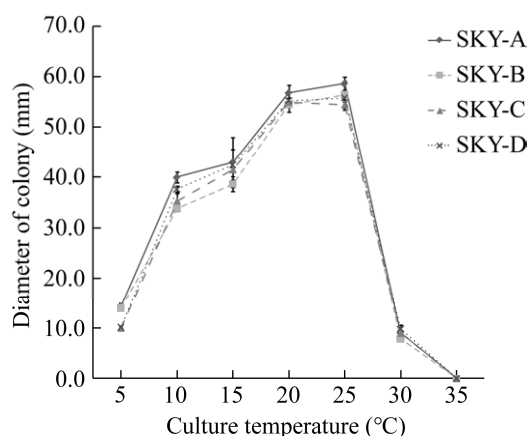


图3 温度对试验菌株菌丝生长的影响 PDA 黑暗培养 48 h

Figure 3 Effect of temperature on mycelial growth of four *Botrytis cinerea* isolates. Incubated for 48 h on PDA in the dark.

试验菌株在 PDA 平板上 15 °C 和 20 °C 培养 14–40 d, 可以产生两种类型的微菌核及假微菌核: I 型微菌核红褐色至黑褐色, 表生或埋生, (26.9–492.5) $\mu\text{m} \times$ (14.9–149.3) μm , 微菌核的组成细胞大小为 (12.7–35.0) $\mu\text{m} \times$ (11.9–25.3) μm ; II 型微菌核近黑色, 埋生, (35.8–373.1) $\mu\text{m} \times$ (23.9–229.9) μm , 微菌核的组成细胞大小为 (8.2–16.4) $\mu\text{m} \times$ (8.2–14.9) μm ; 假微菌核由特化的附着胞组成, 暗褐色至近黑色, 埋生, (32.0–447.8) $\mu\text{m} \times$ (19.4–358.2) μm 。微菌核和假微菌核的产生时间和数量在菌株和重复间也存在较大差异。两种类型的微菌核及假微菌核的培养特征用肉眼很难区分开来, 但它们的显微形态特征差别明显(图 6–8)。

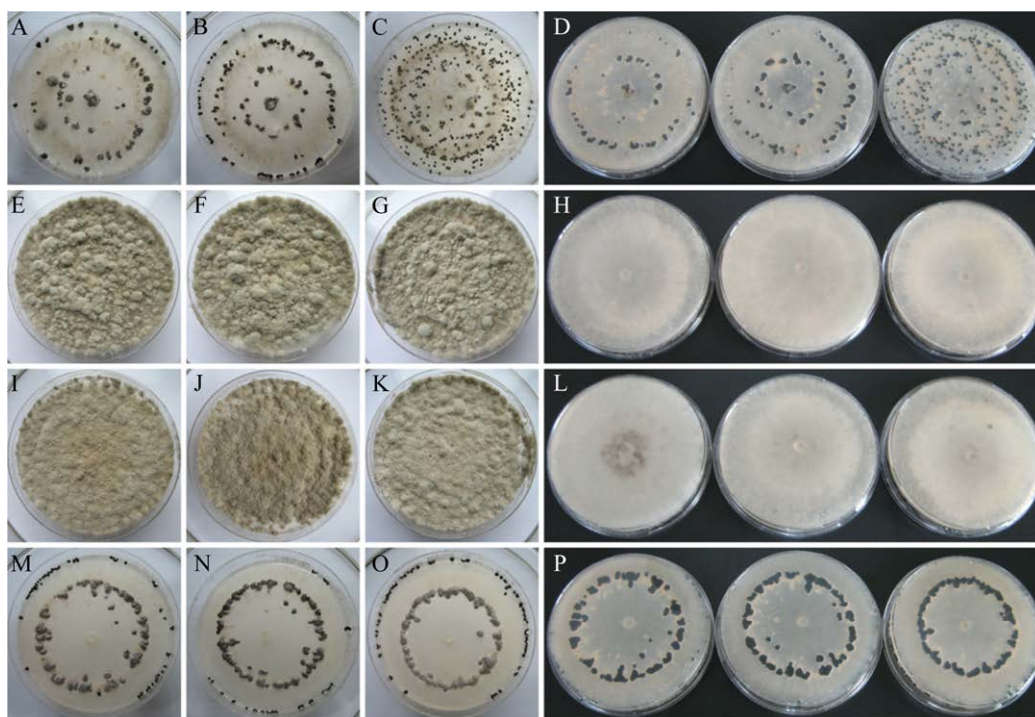


图4 试验菌株在 PDA 平板上 20 °C 黑暗培养 14 d 的菌落形态 A–D: 菌株 SKY-A (培养物正面和背面); E–H: 菌株 SKY-B (培养物正面和背面); I–L: 菌株 SKY-C (培养物正面和背面); M–P: 菌株 SKY-D (培养物正面和背面). 培养皿直径: 90 mm

Figure 4 Colony characteristics of four *Botrytis cinerea* isolates grown on PDA for 14 d at 20 °C in the dark. A–D: Cultures (front and back) of SKY-A. E–H: Cultures (front and back) of SKY-B. I–L: Cultures (front and back) of SKY-C. M–P: Cultures (front and back) of SKY-D. Diameter of Petri dishes: 90 mm.

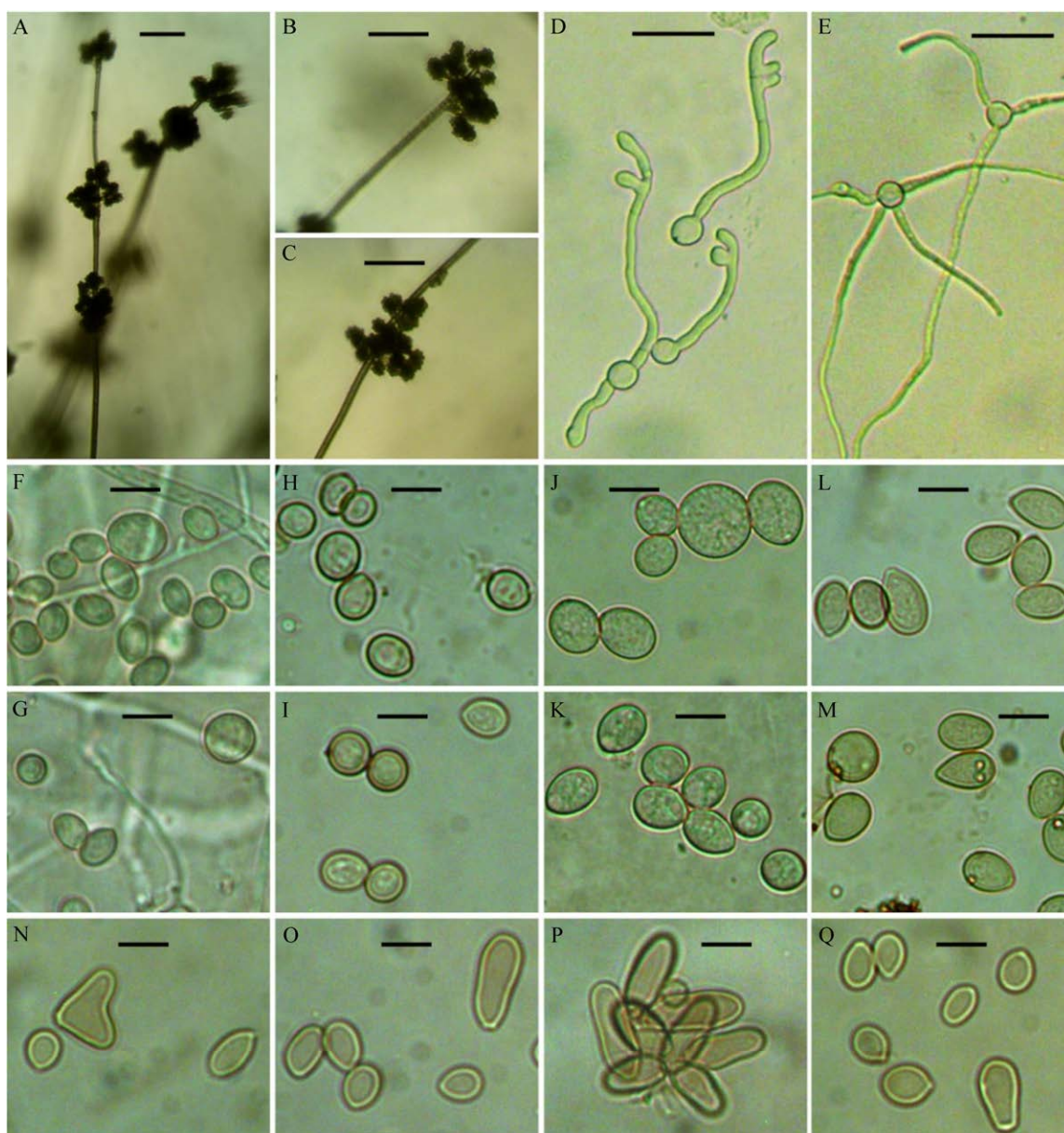


图5 试验菌株的分生孢子梗、分生孢子形态及孢子萌发特征 A-C: 分生孢子梗; D、E: 分生孢子
在载玻片上的水滴中 20 °C、24 h 萌发产生 1-4 根芽管; F-Q: 分生孢子. A-C、F、G、N-Q: 菌株 SKY-A;
H、I: 菌株 SKY-B; D、E、J、K: 菌株 SKY-C; L、M: 菌株 SKY-D. A-C、F-I、K-M: 在 PDA 平板上
20 °C 黑暗培养 10 d; J、N-Q: 在 PDA 平板上 15 °C 黑暗培养 21 d. 标尺: A-C, 100 μ m; D、E,
40 μ m; F-Q, 10 μ m

Figure 5 Morphological characters of conidiophores, conidia and germinated conidia of four *Botrytis cinerea* isolates. A-C: Well-developed macroconidiophores. D and E: Germinated conidia with 1-4 germ tubes, when conidia incubated for 24 h in water drops at 20 °C on slide. F-Q: Macroconidia. A-C, F, G, and N-Q: Isolate SKY-A. H and I: Isolate SKY-B. D, E, J, and K: Isolate SKY-C. L and M: Isolate SKY-D. A-C, F-I, and K-M: Grown on PDA for 10 d at 20 °C in the dark. J and N-Q: Grown on PDA for 21 d at 15 °C in the dark. Scale: A-C, 100 μ m; D and E, 40 μ m; F-Q, 10 μ m.

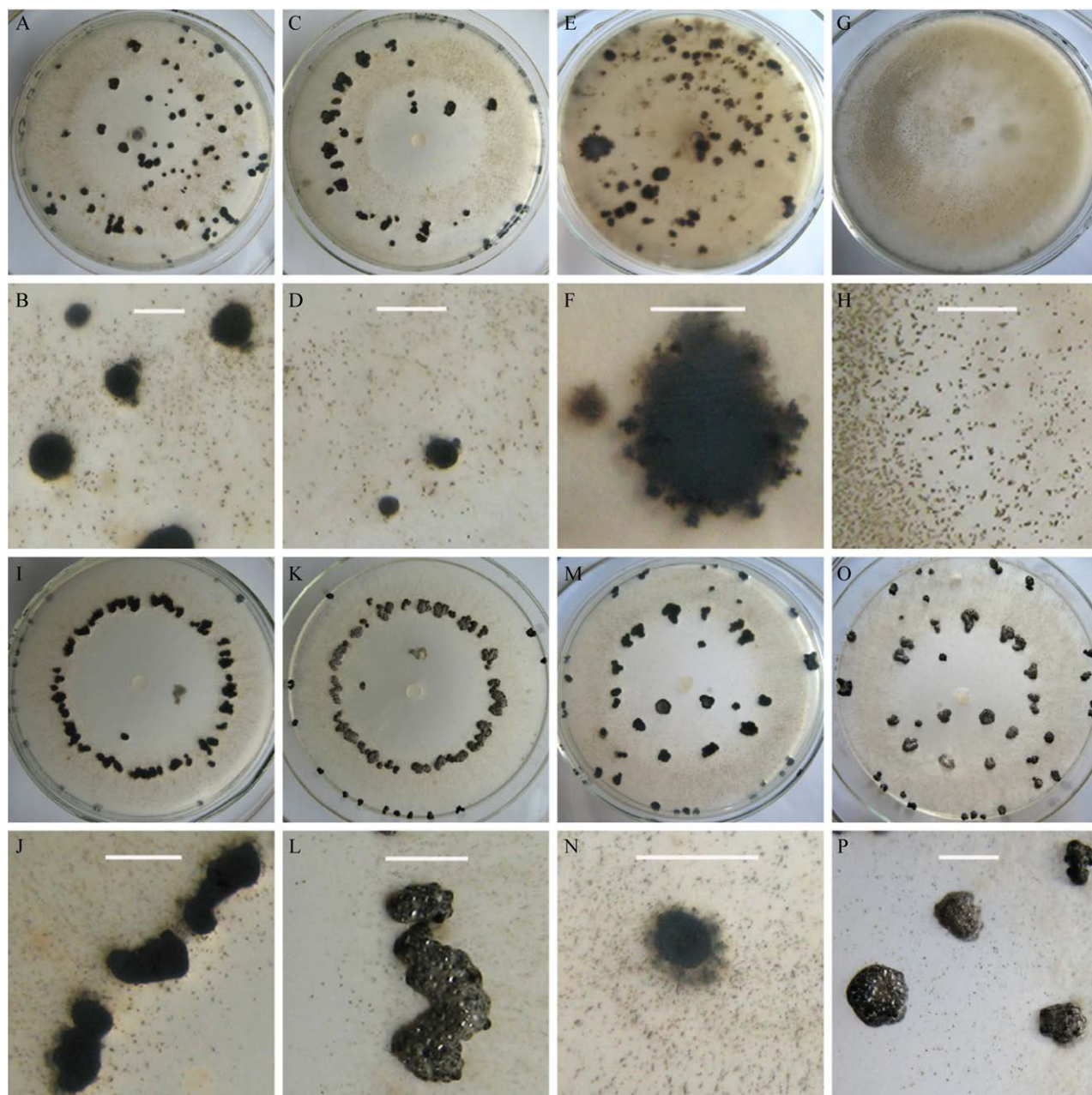


图6 试验菌株在PDA平板上20℃黑暗培养14–40 d产生的菌核、微菌核和假微菌核 A–D: 菌株SKY-A(培养物背面), 培养21 d; E、F: 菌株SKY-B(培养物背面), 培养40 d; G、H: 菌株SKY-C(培养物背面), 培养14 d; I–P: 菌株SKY-D, 培养物背面(I、J、M、N)和正面(K、L、O、P), 培养21 d(I–L)和40 d(M–P)。标尺: 5 mm。培养皿直径: 90 mm

Figure 6 Morphological features of sclerotium, microsclerotium and pseudo-microsclerotium produced by four *Botrytis cinerea* isolates grown on PDA for 14–40 d at 20 °C in the dark. A–D: Cultures (back) of isolate SKY-A, incubated for 21 d. E, F: Culture (back) of isolate SKY-B, incubated for 40 d. G, H: Culture (back) of isolate SKY-C, incubated for 14 d. I–P: Cultures of isolate SKY-D, back (I, J, M, and N) and front (K, L, O, and P), incubated for 21 d (I–L) and 40 d (M–P). Scale bar: 5 mm. Diameter of Petri dishes: 90 mm.

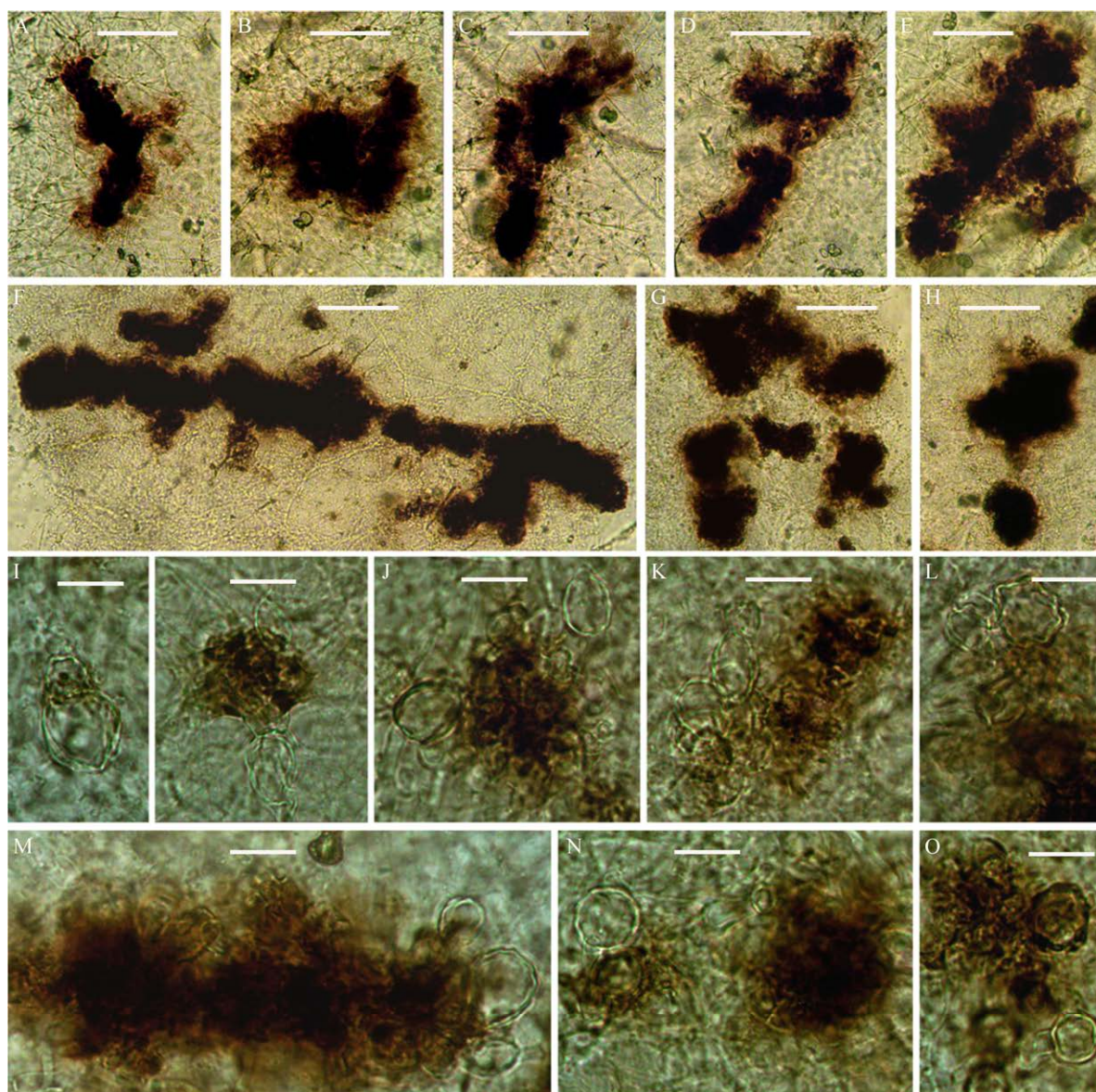


图7 I型微菌核的显微形态特征(PDA 黑暗培养) A-H: 成熟的微菌核; I-O: 发育中的微菌核. A-E: 菌株 SKY-A, 15 °C下培养 21 d; F-O: 菌株 SKY-D, 20 °C下培养 40 d. 标尺: A-H, 100 μ m; I-O, 20 μ m
Figure 7 Microscopic features of type-I microsclerotium of *Botrytis cinerea* (grown on PDA in the dark). A-H: Mature microsclerotia. I-O: Developing microsclerotia. A-E: Isolate SKY-A, incubated for 21 d at 15 °C. F-O: Isolate SKY-D, incubated for 40 d at 20 °C. Scale bar: A-H, 100 μ m; I-O, 20 μ m.

2.5 热胁迫对病菌产生菌核的诱导作用

30 °C培养 7 d, 再转至 20 °C培养 14 d, 可诱导菌株 SKY-B 和 SKY-C 产生菌核, 同时产生较初始分离物明显更多的分生孢子; 变异菌株的产菌核及产孢能力在继代培养中可遗传。

在产生菌核的培养物的培养基中可见到明显至不甚明显的淡紫红色色素(图 9, 图 10)。

通过与文献中描述的相关真菌的形态特征进行比对^[5,18-20], 将 4 个试验菌株鉴定为灰葡萄孢菌(*B. cinerea* Pers.)。

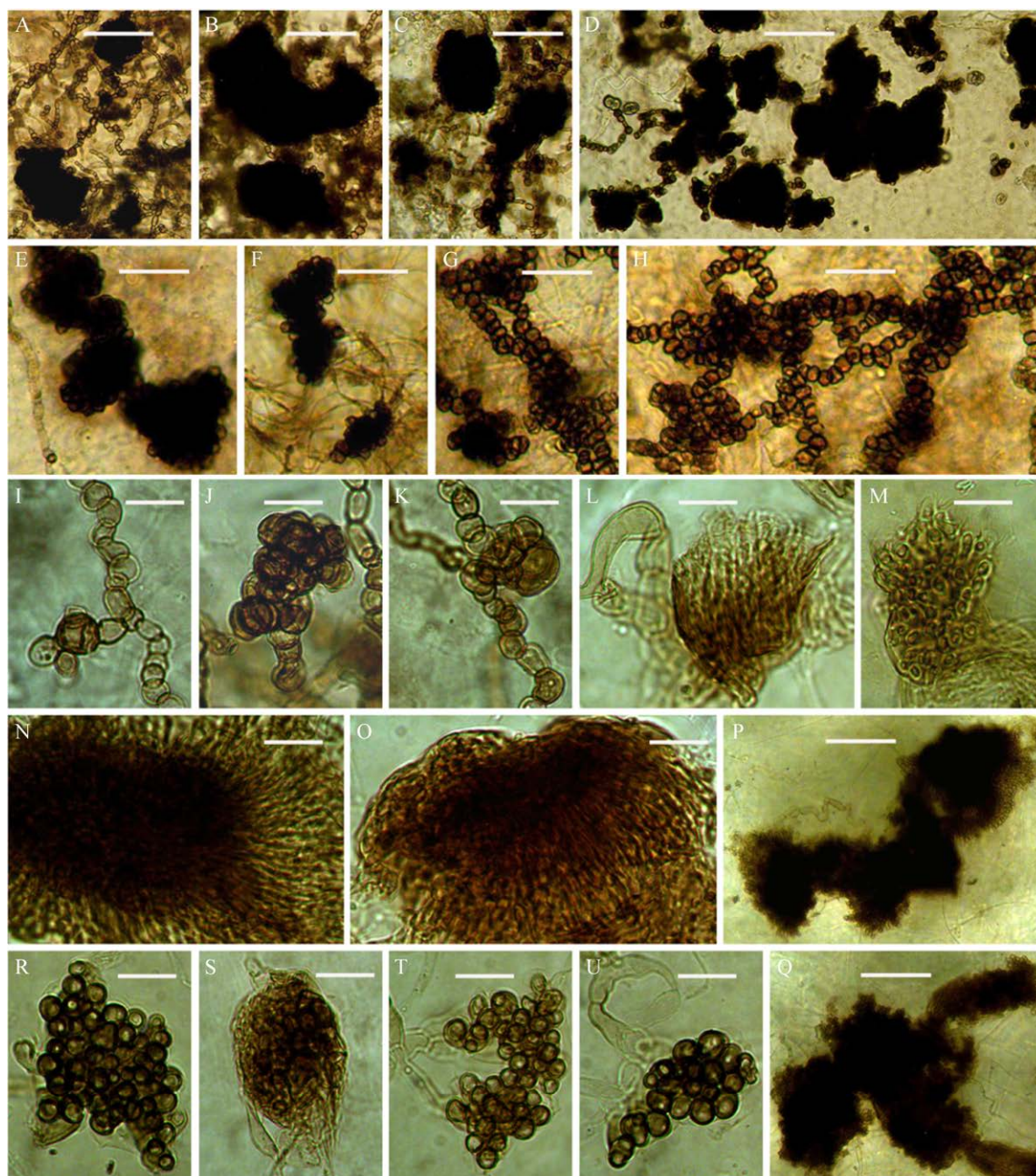


图 8 II型微菌核和假微菌核的显微形态特征(PDA 黑暗培养) A-K: II型微菌核, 菌株 SKY-B, 15 °C 培养 21 d (A-F, 成熟的微菌核; G-K, 发育中的微菌核); L-U: 假微菌核(L-Q, 菌株 SKY-C, 20 °C 培养 40 d; R-U, 菌株 SKY-A, 20 °C 培养 40 d). 标尺: A-D、P、Q, 100 μ m; E-H, 50 μ m; I-O、R-U, 20 μ m

Figure 8 Microscopic features of type-II microsclerotium and pseudo-microsclerotium of *Botrytis cinerea* (grown on PDA in the dark). A-K: Type-II microsclerotium, isolate SKY-B incubated for 21 d at 15 °C (A-F: Mature microsclerotia; G-K: Developing microsclerotia). L-U: Pseudo-microsclerotium (L-Q: Isolate SKY-C, incubated for 40 d at 20 °C; R-U: Isolate SKY-A, incubated for 40 d at 20 °C). Scale bar: A-D, P and Q, 100 μ m; E-H, 50 μ m; I-O and R-U, 20 μ m.

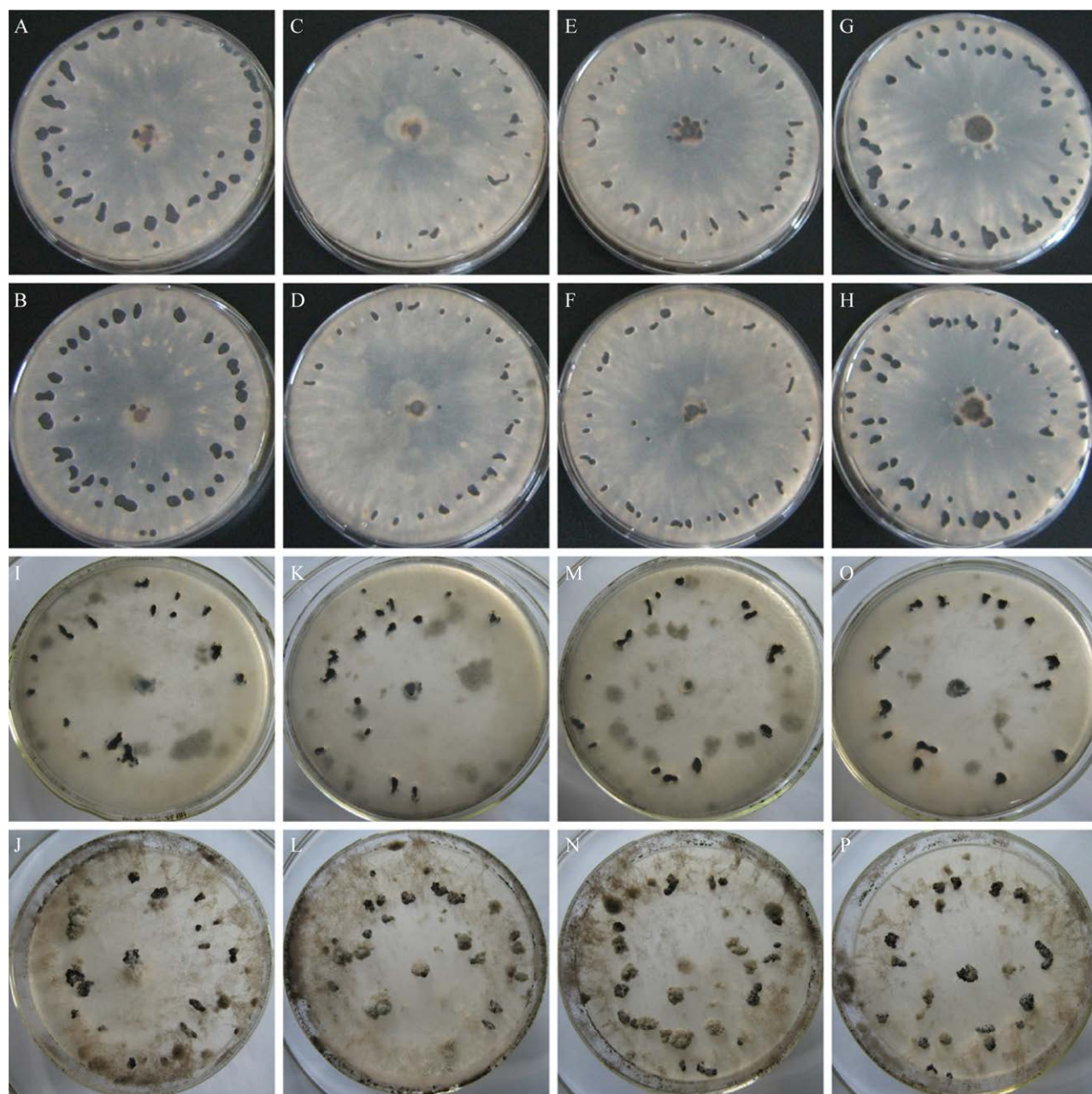


图9 热胁迫诱导产生菌核及其可遗传性(I) A-H: 初始分离物在 PDA 平板上 30 °C 黑暗培养 7 d 后, 转至 20 °C 培养 14 d, 培养物背面(A、B: 菌株 SKY-A; C、D: 菌株 SKY-B; E、F: 菌株 SKY-C; G、H: 菌株 SKY-D)。I-P: 变异菌株的继代培养物在 PDA 平板上 20 °C 黑暗培养 21 d (I-L: SKY-B; M-P: SKY-C; I、K、M、O: 培养物背面; J、L、N、P: 培养物正面)。培养皿直径: 90 mm

Figure 9 Sclerotia-forming induced by heat stress and its heritability (I). A-H: Original isolates of *Botrytis cinerea* grown on PDA for 7 d at 30 °C in the dark, then for 14 d at 20 °C, cultures back (A and B: Isolate SKY-A; C and D: Isolate SKY-B; E and F: Isolate SKY-C; G and H: Isolate SKY-D). I-P: Subcultures of mutants incubated for 21 d at 20 °C in the dark (I-L: Isolate SKY-B; M-P: Isolate SKY-C; I, K, M and O: Cultures back; J, L, N and P: Cultures front). Diameter of petri dishes: 90 mm.

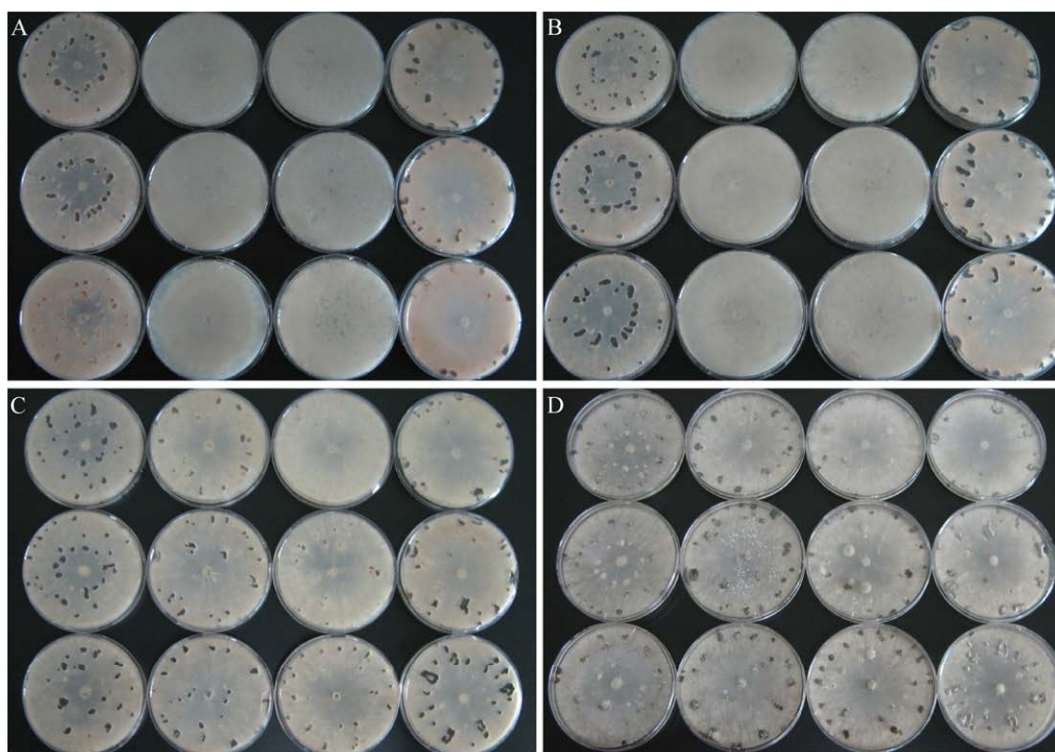


图 10 诱变菌株产生菌核能力的可遗传性(II) A、B: 初始分离物在 PDA 平板上 15 °C 黑暗培养 14 d (A) 和 21 d (B), 培养物背面. C、D: 变异菌株(由热胁迫诱导)的继代培养物在 PDA 平板上 15 °C 黑暗培养 21 d, 培养物背面(C)和正面(D). A–D 从左至右第 1–4 列依次是菌株 SKY-A、SKY-B、SKY-C 和 SKY-D. 培养皿直径: 90 mm

Figure 10 The heritability of sclerotia formation ability of mutants (II). A and B: Original isolates of *Botrytis cinerea* grown on PDA for 14 d (A) and 21 d (B) at 15 °C in the dark, cultures back. C and D: Subcultures of mutants (induced by heat stress) incubated for 21 d on PDA at 15 °C in the dark, back (C) and front (D). A–D: Columns 1 through 4 (left-to-right) are, in order, isolate SKY-A, SKY-B, SKY-C and SKY-D. Diameter of petri dishes: 90 mm.

2.6 病原菌的分子生物学鉴定结果

用于本研究的 4 个 *B. cinerea* 菌株的基础信息及 NMDC 序列登录号见表 4。

BLASTn 分析结果显示, 4 个试验菌株的 rDNA-ITS 序列与 GenBank 中已知且有可靠文献支撑的 *B. cinerea* 序列相似性达 99.8%–100.0%。

在以最大似然法和最大简约法构建的 *g3pdh*、*hsp60* 和 *rpb2* 多基因联合系统发育树中, 4 个试验菌株均与 *B. cinerea* 菌株聚在一起, 并展示出较紧密的寄主(菊科)和地域(甘肃)关联

(图 11)。分子生物学鉴定结果与形态学鉴定结果一致。

3 讨论与结论

经致病性测定、形态学和分子生物学鉴定, 确认灰葡萄孢菌(*B. cinerea*)是引起兰州市食用向日葵叶斑和盘腐的病原菌。*B. cinerea* 引起向日葵灰霉病在我国西北地区系首次报道。

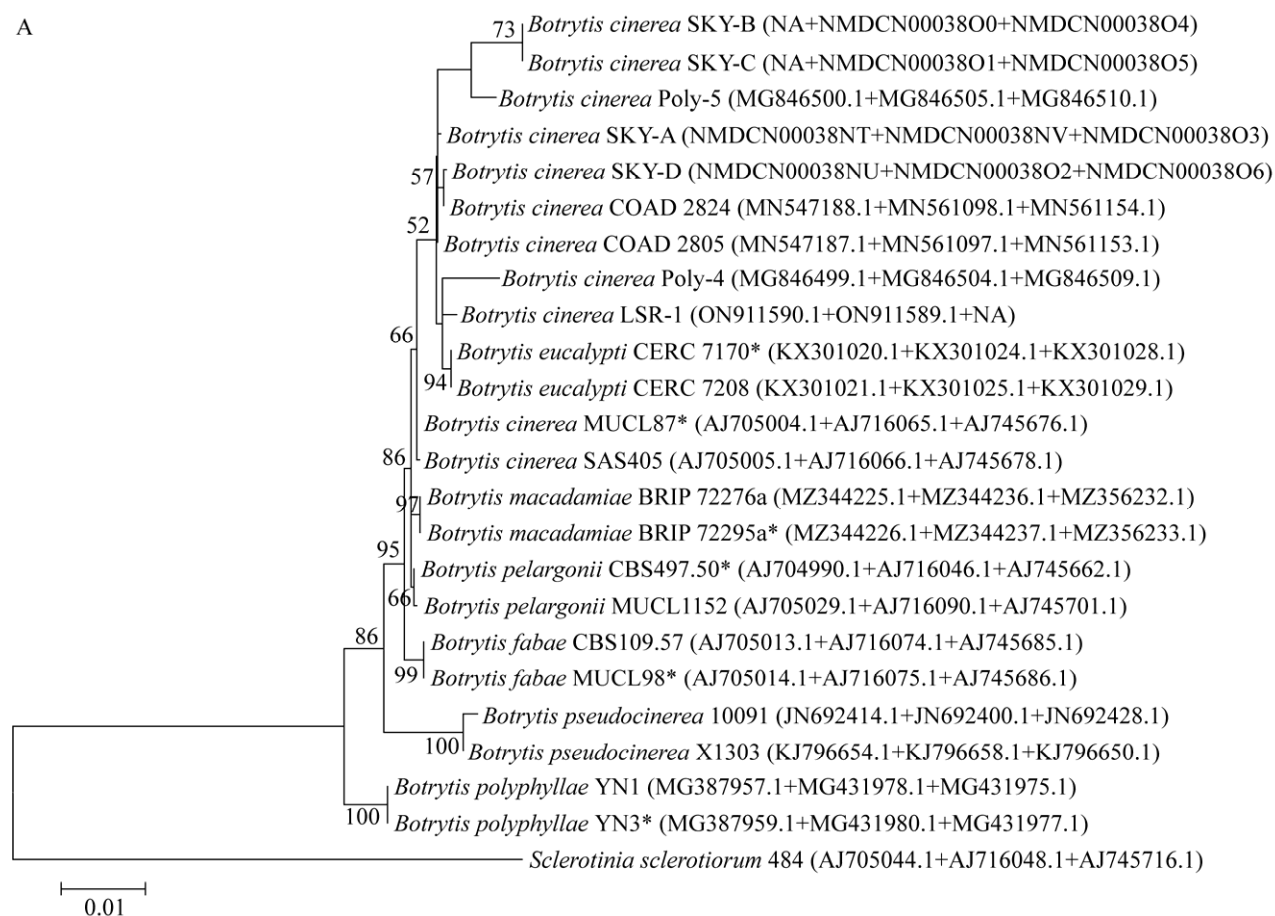
B. cinerea 在常态下产生分生孢子和菌核, 大型分生孢子含有 1–12 个细胞核(平均 3.4 个),

表 4 用于本研究的 4 个 *Botrytis cinerea* 菌株的基础信息及 NMDC 序列登录号Table 4 Origin and NMDC accession numbers of the sequences for four *Botrytis cinerea* isolates in this study

菌株 编号 Strain No.	寄主 Host	来源 Source	地域 Locality	采集日期 Collection date	ITS	<i>g3pdh</i>	<i>hsp60</i>	<i>rpb2</i>
SKY-A	<i>Helianthus annuus</i>	leaf (sample No. 1)	China: Gansu, Lanzhou	2022-08-19	NMDCN00038NP	NMDCN00038NT	NMDCN00038NV	NMDCN00038O3
SKY-B	<i>Helianthus annuus</i>	head (sample No. 2)	China: Gansu, Lanzhou	2022-09-27	NMDCN00038NQ	—	NMDCN00038O0	NMDCN00038O4
SKY-C	<i>Helianthus annuus</i>	head (sample No. 2)	China: Gansu, Lanzhou	2022-09-27	NMDCN00038NR	—	NMDCN00038O1	NMDCN00038O5
SKY-D	<i>Helianthus annuus</i>	head (sample No. 3)	China: Gansu, Lanzhou	2022-09-27	NMDCN00038NS	NMDCN00038NU	NMDCN00038O2	NMDCN00038O6

核酸序列数据存储在国国家微生物科学数据中心(NMDC): <https://nmhc.cn/resource/genomics/sequence>Data is deposited in National Microbiology Data Center (NMDC): <https://nmhc.cn/resource/genomics/sequence>.

A



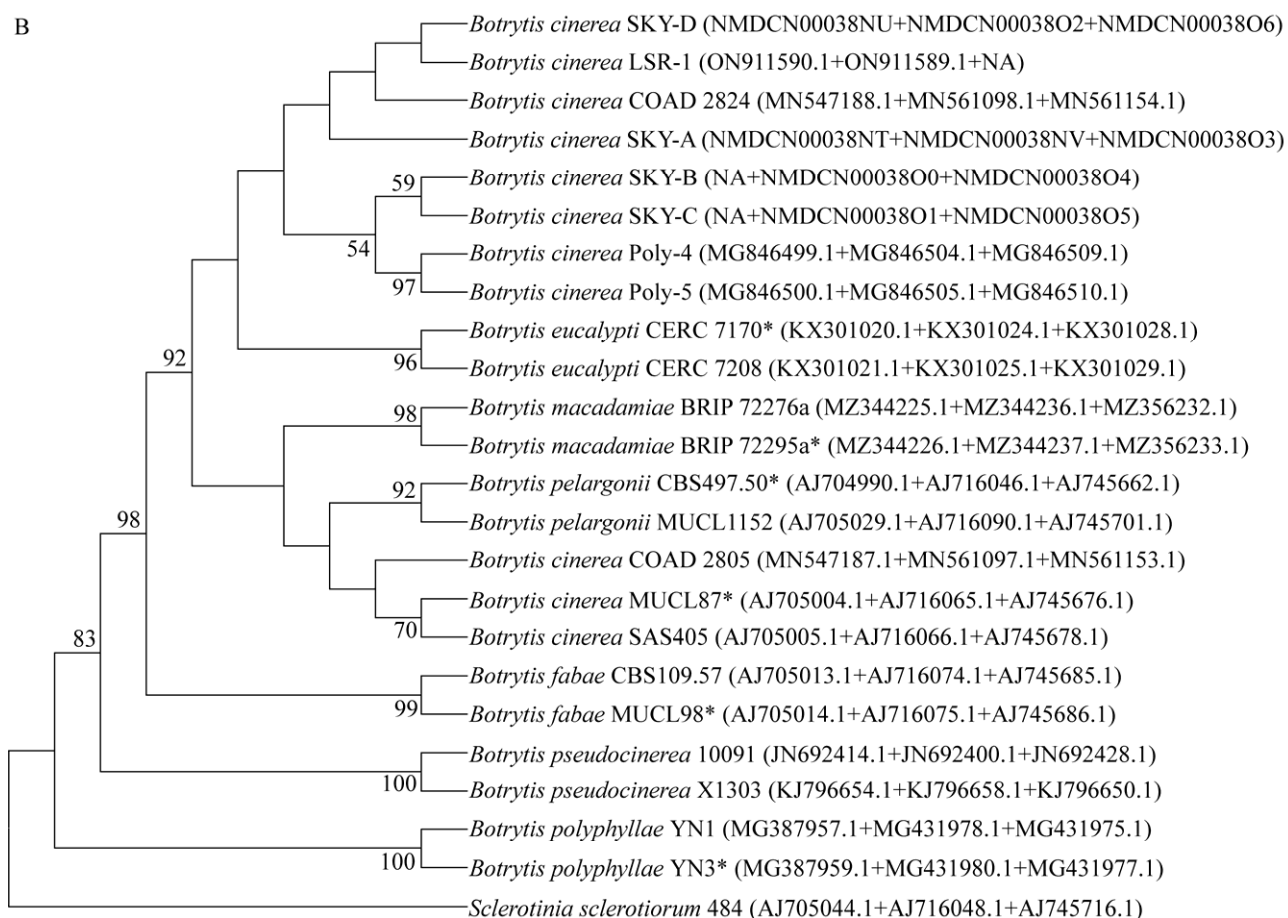


图 11 基于 *g3pdh*、*hsp60* 和 *rpb2* 基因序列以最大似然法(A)和最大简约法(B)构建的系统发育树。分支上的数据表示 bootstrap 检验的支持百分率，自展支持值(bootstrap)>50%则显示在各个进化分支节点上；以 *Sclerotinia sclerotiorum* 为外群；模式菌株在培养物编号后标注以星号；括号内 GenBank 或 NMDC 登录号顺序为 *g3pdh+hsp60+rpb2*，NA 表示无登录号；图中标尺表示每个位点的预期碱基替代数量

Figure 11 Phylogenetic trees based on combined *g3pdh*, *hsp60* and *rpb2* gene sequences using maximum likelihood (A) & maximum parsimony (B) methods. The confidence values over 50% from 1 000 replicate bootstrap samplings are shown at each node. *Sclerotinia sclerotiorum* is used as the out group. Type strains are marked with an asterisk after the culture number. The order of GenBank or NMDC accession number in parentheses is *g3pdh+hsp60+rpb2*, and the NA indicates no accession number. The scale bar shows the expected number of changes per site.

在琼脂培养基上容易萌发；小型分生孢子单核，在有性生殖中起精子的作用，在琼脂培养基上很少萌发；菌核可以抵抗恶劣的环境条件，在条件适宜时产生新鲜的菌落和孢子^[19]。Urbasch 在培养中和寄主植物上观察到 *B. cinerea* 可产生初无色，老化后淡黄色至褐色，单细胞[大小：

(10.0–30.0) $\mu\text{m} \times$ (5.0–25.0) μm]或多细胞[大小：(12–35) $\mu\text{m} \times$ (6–30) μm]的厚垣孢子，厚垣孢子萌发后直接或产生大型分生孢子成为再次侵染的接种体，引起新的感染^[21–22]。

红色色素在灰葡萄孢菌中比较罕见，Plesken 等 2012–2013 年从单子叶植物黄花鸢尾(*Iris*

pseudoacorus L.)的花和叶片上分离到 22 株灰葡萄孢菌株,其中的 21 株在麦芽琼脂培养基上培养时产生红色色素,这些红色菌株含有编码合成比卡菌素(bikaverin)的聚酮合成酶基因 *bcbikI*^[20];刘帆兴等 2021 年从莴苣上分离到一株能够产生红色分泌物的灰葡萄孢菌株^[10]。比卡菌素具有抗生素、抗癌和抗卵菌活性,这种色素是否对灰葡萄孢菌具有选择性优势尚不明确^[20]。在本研究中,试验菌株紫红色分泌物的产生似乎与菌核形成相关联。

Barnes 通过高温(48–80 °C)处理灰葡萄孢菌的分生孢子,观察到处理后存活孢子形成的菌落发生的形态变异,包括产生粉红色菌核、粉红色的菌丝体和粉红色的附着胞;培养一周左右的菌丝可能转化为厚垣孢子链,或在基内生菌丝体的短侧枝上产生厚垣孢子;在相同处理的不同重复间,菌核产生与否,及菌核丰富度差异巨大,非常不稳定;在老化的培养中,以及在相对较高温下(≥ 30 °C)培养,野生型菌株显示出异常生长的趋势(培养物的气生菌丝体较多,分生孢子产量大幅减少,形成许多粉红色的附着胞,培养基经常染色,很少产生菌核),但这些变异不能传递给继代培养物^[23]。在本研究中,试验菌株在 30 °C 培养 7 d,再转至 20 °C 培养 14 d,可诱导 2 个不产菌核的菌株产生菌核和更多的分生孢子,变异菌株的产菌核及产孢能力可在继代培养中遗传。

B. cinerea 是多寄主死体营养型病菌,由于这种真菌在世界范围内的重要性和分子遗传工具的可用性,它已成为研究最广泛的坏死性真菌病原菌,并成为坏死性真菌分子研究的重要模型菌^[4]。*B. cinerea* 具有丰富的表型特征和遗传多样性,且群体内遗传多样性远远高于群体间,其多样性与寄主植物、地理来源、气候条件等具有一定的关系,但不同研究者得出的结

论不同^[24]。在本研究构建的 *g3pdh*、*hsp60* 和 *rpb2* 多基因联合系统发育树中,4 个试验菌株与 *B. cinerea* 菌株聚在一起,并展示出较紧密的寄主(菊科)和地域(甘肃)关联,研究结果支持 Azevedo 等^[9]及 Garfinkel^[25]的推论,即桉葡萄孢(*B. eucalypti* Liu & Chen)和天竺葵葡萄孢(*B. pelargonii* Røed)很可能是 *B. cinerea* 的单倍型;依据研究结果,澳洲坚果葡萄孢(*B. macadamiae* Prasannath, Akinsanmi & Shivas)也应归入 *B. cinerea*。

在过去的 10 年中,许多参与 *B. cinerea* 发育和致病性的基因已经被表征;在 *B. cinerea* 基因组中编码了近 11 000 个基因,截至目前,通过突变分析只对其中的不到 150 个基因进行了遗传研究,这也说明我们对这种真菌的了解还相当有限^[26]。

菌核(sclerotia)和微菌核(microsclerotia)是一些子囊菌和担子菌在特殊条件下形成的具有抵御恶劣环境能力的休眠结构,在真菌生活史及病原真菌的侵染循环中具有重要意义^[27-28]。大丽轮枝菌(*Verticillium dahlia* Kleb.)的微菌核通常被认为起源于单个或连续的菌丝,这些菌丝膨大并形成多隔细胞,随后横向出芽产生球形细胞簇,之后这些细胞着色^[29]。由于 *V. dahlia* 具有形成黑化微菌核的能力,在没有宿主的情况下,可以在土壤中存活 14 年^[30]。本研究中观察到的试验菌株产生的Ⅱ型微菌核与 *V. dahlia* 的微菌核的形成方式有部分相似;两种类型的微菌核在 *B. cinerea* 中均是首次发现。*B. cinerea* 微菌核产生的分子机制、抗逆能力及其在病害侵染循环中的作用等都有待开展进一步的试验研究。

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