

核桃腐烂病拮抗菌 *Pseudomonas chlororaphis* 的筛选、鉴定及其防病促生效果

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摘要:【目的】为有效控制核桃腐烂病的发生与发展, 从新疆温宿县核桃园土壤中分离既对核桃腐烂病有很好防效, 又对核桃种子萌芽具有促进作用的拮抗细菌。【方法】通过稀释涂布平板法进行分离培养、平皿对峙筛选, 通过形态学和分子生物学方法进行鉴定, 以及通过室内离体枝条、盆栽和田间盆栽试验探究防病促生作用。【结果】筛选出1株生防菌WS-04, 通过形态学观察、生理生化特性测定及分子鉴定, 最终确定菌株WS-04为绿针假单胞菌 *Pseudomonas chlororaphis*。研究发现WS-04能使病原菌菌丝发生断裂和解体, 使菌丝无法正常生长, 对核桃腐烂病菌 *Cytospora chrysosperma* 的抑菌效果达87.00%, 其发酵液对核桃腐烂病的防效也达到84.96%, 还具有一定的热稳定性。经过拮抗菌株发酵滤液浸泡处理的核桃种子, 萌芽率和发芽率与对照相比都有显著提高($p<0.05$), 且有效地降低了坏种率。菌株发酵滤液能促进核桃幼苗苗高、主根长、须根数、叶片面积和干质量的显著增加, 其依次增加了25.40%、83.33%、48.02%、24.76%和98.40%。【结论】从核桃园土壤中分离并筛选出一株具有广谱抗菌且对核桃种子和幼苗具有促生作用的生防细菌。研究成果为核桃腐烂病的生物防治提供了新的菌株来源。

关键词:核桃腐烂病; 拮抗细菌; 促生作用

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Screening and identification of antagonistic bacterium *Pseudomonas chlororaphis* for walnut rot disease and its effect on promoting germination of seeds and growth of seedlings in walnut

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Abstract:【Objective】The study aimed to select indigenous bacterial strain that could control walnut rot and other fungal diseases and promote the germination of seeds and growth of seedlings of walnut. 【Methods】Bacteria were isolated from the rhizosphere soil of walnut (*Juglans regia* L.) trees using confronting plate methods and streaked on agar to screen for antagonistic strains. The broad-spectrum antibacterial activity of the selected antagonistic strains against walnut rot disease was evaluated. The morphological characteristics, physicochemical properties, and molecular biological characters were employed to identify the screened strains. The thermal stability of the antagonistic strains was determined using mycelial growth assays under different temperature gradients. The inoculation experiments

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were conducted to ascertain the effects of the antagonistic strains on the walnut rot symptoms. The pot experiments in a controlled environment were carried out to analyze the impact of varying concentrations of fermented filtrate on the germination of walnut seeds and the rate of poor-quality seeds. The mature, unspoiled walnuts weighing approximately 12 grams each were selected as experimental materials and dried in the air naturally. The fermented filtrate of the antagonistic strain WS-04 was diluted by sterilized distilled water, and five concentrations ($50, 150, 250, 350$, and $450 \text{ mg} \cdot \text{mL}^{-1}$) were set for treatment. The sterilized distilled water was used as a control, and each concentration was applied to 20 seeds with three repeats for each experiment. Prior to soaking, the walnut shells were opened to ensure that the kernels would contact the fermented filtrate. After soaking for one day, the seeds were rinsed with clean water and placed in a humid germination box within an incubator at a constant temperature of 30°C to promote germination. Daily observations were made during the course of seed germination, and any rotten or moldy seeds were promptly removed. The germinated seeds were transplanted into pots with a diameter of 20 cm, with two seeds planted per hole, covered with 1 cm of substrate (vermiculite: perlite: peat soil: soil in a volume ratio of 1:1:1:1). The number of germinated seeds was recorded, and the rate of blanching was calculated. Following the emergence of two leaves, the germination rate and the rate of poor-quality seeds were calculated. Simultaneously, both indoor pot experiments and field pot experiments were conducted to study the effect of different concentrations of the fermented filtrate on the growth of walnut seedlings. The pretreatment of hulled walnut seeds for the experiment of promotion of growth on seedlings was as follows: the walnuts were gently split open to ensure the contact between the kernel and the fermented filtrate of the antagonistic bacteria. A concentration of $150 \text{ mg} \cdot \text{mL}^{-1}$ of the antagonistic culture was assigned to the experimental group, while the control group consisted of a medium without antagonistic culture; each group comprised 25 walnut seeds, with five repeats. After germination, the single well plates (32 wells, 6 cm \times 4.5 cm) were used for the treatments, one seed per well. The seedling treatment in plates involved sowing in the wells and initiating irrigation with the antagonistic filtrate (experimental group received $150 \text{ mg} \cdot \text{mL}^{-1}$ of fermented filtrate), administering 50 mL every five days. After 20 days of growth, the seedlings from the well plates were transplanted outdoors. For the pot experiments, the walnut seedlings were transplanted into experimental plots (with a diameter of 40 cm), with each pot irrigated with 500 mL of fermented filtrate (once every ten days). After 90 days of transplanting, five plants were randomly selected to measure biological indicators, including dry weight, seedling height, main root length, and root count. **【Results】** A total of 157 bacterial strains were isolated from walnut rhizosphere soil, of which five strains were found to inhibit walnut rot disease, with strain WS-04 demonstrating an inhibition rate of 87.00%. The inhibition rates for *Cytospora nivea*, *Valsa mali*, *Valsa ambiens*, *Cytospora chrysosperma*, *Cytospora leucostoma*, *Alternaria alternata*, *Verticillium dahliae*, and *Fusarium oxysporum* were all above 70.00%. Through the morphological characteristics, physicochemical properties, and molecular biological confirmation, the strain WS-04 was ultimately identified as *Pseudomonas chlororaphis*. The antibacterial activity of *C. chrysosperma* was assessed using the mycelial growth rate method, revealing that WS-04 exhibited significant inhibitory effects on *C. chrysosperma*, with inhibition rates exceeding 80% as the concentration of fermented filtrate increased. At a concentration of 15%, the inhibition rate reached 93.65%. The thermal stability assays indicated that as the temperature increased, the inhibition rate of the WS-04 gradually declined; however, it remained above 85% against *C. chrysosperma*, indicating favorable thermal stability. The treatment at 95°C for 30 minutes demonstrated that increased temperature progressively led to the loss of the activity of the fermented filtrate. The isolation protection tests suggested that the

WS-04 would have good preventive effects on anti-walnut rot, with an average disease lesion area of 0.85 cm^2 for treated samples compared with 6.32 cm^2 for the control group, resulting in a control efficacy of 84.96%. The pot experiments revealed that the germination rate of the walnut seeds was improved with increase of the concentrations after treatments. At concentrations of $150 \text{ mg} \cdot \text{mL}^{-1}$ and $250 \text{ mg} \cdot \text{mL}^{-1}$, significant promotion of walnut seed germination was observed. The germination rate was notably higher in the treatment groups with concentrations of $250 \text{ mg} \cdot \text{mL}^{-1} > 150 \text{ mg} \cdot \text{mL}^{-1} > 350 \text{ mg} \cdot \text{mL}^{-1} > 50 \text{ mg} \cdot \text{mL}^{-1} > 450 \text{ mg} \cdot \text{mL}^{-1}$ compared with the control group ($p < 0.05$). As the concentration increased, the germination rate initially rose and then declined; within the range of 50 to $250 \text{ mg} \cdot \text{mL}^{-1}$, the germination rate of walnut seeds steadily increased, but when the concentration of the fermented filtrate exceeded $250 \text{ mg} \cdot \text{mL}^{-1}$, the germination rate began to decrease. At a concentration of $150 \text{ mg} \cdot \text{mL}^{-1}$, the percentage of the poor-quality seeds was the lowest, reaching 6.68%. When the concentration of the antagonistic bacteria exceeded $150 \text{ mg} \cdot \text{mL}^{-1}$, the rate of poor-quality seeds exhibited an upward trend; however, the rates of poor quality seeds of all concentrations remained significantly lower than those in the control group ($p < 0.05$). In the field experiments, we found that the WS-04 could promote the growth of walnut seedlings, increasing seedling height, root length, leaf area, and dry weight by 25.40%, 83.33%, 48.02%, 24.76%, and 98.40%, respectively. 【Conclusion】 A biocontrol bacterium with broad antibacterial spectrum and growth promotion effect on walnut seeds and seedlings was isolated and screened from the soil of walnut orchard. This would provide a novel strain source for the biological control of the walnut rot disease.

Key words: Walnut rot disease; Antagonistic bacteria; Promoting effect

核桃(*Juglans regia* L.)是胡桃科核桃属的重要经济树种,是世界四大干果之一,具有较高营养价值和药用价值^[1]。新疆是中国核桃种植第二大省份,2020年新疆种植面积已达到 $4.14 \times 10^5 \text{ hm}^2$,年产量达到 $1.15 \times 10^6 \text{ t}$ ^[2]。近年来随着新疆核桃的大面积种植,核桃腐烂病大面积发生,严重制约核桃产业的健康发展^[3-5]。目前生产上病害仍然以化学防治为主,但化学杀菌剂存在易污染环境、对人畜不安全、农药残留等一系列问题^[6-7]。因此,开发出高效的生防菌株替代化学药剂防治核桃腐烂病,不仅可以有效控制病害的发生与发展,还可以有效规避化学防治带来的系列问题。

土壤细菌是植物根际土壤微生态的优势种群之一,具有分布广、数量多、营养要求简单、繁殖快、竞争定殖力强等特点^[8]。土壤细菌能产生吩嗪-1-羧酸(phenazine-1-carboxylic acid, PCA)、2,4-二乙酰基间苯三酚、硝毗咯菌素(pyrrolnitrin, PRN)、藤黄绿脓菌素(pyoluteorin, PLT)、假单胞菌酸(pseudomonic-acid)、氢氰酸和植物生长素吲哚乙酸(indole-3-acetic acid, IAA)等次生代谢产物,这些有机化合物不仅能增强植物抗病能力,而且还对植物生长发育产生积极影响^[9-10]。目前对于核桃腐烂病的生物防治应

用较多的拮抗细菌主要有芽孢杆菌(*Bacillus* spp.),假单胞杆菌(*Pseudomonas* spp.)、土壤放射杆菌(*Agrobacterium radiobacter*)等。例如许多学者利用假单胞菌成功防治了小麦^[11]、草莓^[12]、小白菜^[13]和番茄^[14]等作物的部分侵染性病害,同时发现假单胞菌还能促进作物的健康生长^[15-16]。魏海雷等^[17]在小麦全蚀病的土壤中分离得到一株荧光假单胞菌2P2 4,通过研究发现此荧光假单胞菌产生的多种抗菌物质对枯黄萎病、全蚀病以及根腐病均有较好的生物防治效果。冉隆贤等^[18]通过筛选发现3株假单胞杆菌及其缺失嗜铁素对桉树灰霉病具有较好的防治效果,且假单胞菌能否产生嗜铁素直接影响桉树灰霉病的发生与否。刘艳萍等^[19]研究表明,恶臭假单胞菌A3产生的嗜铁素可以显著促进黄瓜幼苗的生长。杨艺炜^[20]研究发现绿针假单胞菌XF10能有效地抑制烟草黑胫病菌菌丝生长,且能够抑制游动孢子囊的产生和孢子的萌发,盆栽防效超过70.00%。史娜艳^[21]从核桃树皮分离出一株枯草芽孢杆菌S23,该菌株可以使金黄壳囊孢菌丝发生皱缩、畸形甚至断裂,从而抑制菌丝的生长。展丽然等^[22]从土壤中筛选到一株放线菌Z-6,该菌株对腐烂病菌有较强拮抗作用,经过鉴定该放线菌属于链霉菌属(*Strep-*

tomyces)。

目前对核桃腐烂病的生物防治应用较多的拮抗细菌主要有芽孢杆菌(*Bacillus* spp.)^[23],但是利用绿针假单胞来防治核桃腐烂病还未见报道。如今核桃产业已成为新疆核桃种植区巩固脱贫、促进乡村振兴和增加农民收入的重要支柱性产业。然而核桃腐烂病的发生与蔓延严重阻碍了核桃产业的健康发展。因此,从核桃园土壤中分离筛选活性高、对环境友好且能促进壮苗培育的拮抗菌,对促进新疆核桃产业的健康发展具有重要意义。

1 材料和方法

1.1 试验材料

1.1.1 土壤样品 2023年5月从阿克苏地区温宿县核桃林场采集试验土壤。果园核桃品种为温185,树龄30年,株行距为5 m×7 m。采样时选取长势均匀的核桃树,按照5点采样法采集核桃园土壤,在距离核桃树主干1 m的地方去除0~5 cm表层土,用取土钻采集5~20 cm的土壤,每个30年树龄核桃园采集20份土壤样品,共采集90份样品。按照四分法收集土壤,将土壤过2 mm筛并将土样装入无菌袋中,于4 °C冰箱保存备用。

1.1.2 供试病原菌菌株 核桃腐烂病菌 *Cytospora chrysosperma* 和 *C. nivea*、苹果腐烂病菌 *Valsa mali*、香梨腐烂病菌 *V. ambiens*、沙枣腐烂病菌 *C. chrysosperma*、杏树腐烂病菌 *C. leucostoma*、核桃褐斑病菌 *Alternaria alternata*、棉花黄萎病菌 *Verticillium dahliae*、棉花枯萎病菌 *Fusarium oxysporum*,均由南疆有害生物综合治理兵团重点实验室提供。

1.1.3 培养基 NA培养基:牛肉膏3 g,氯化钠5 g,蛋白胨10 g,琼脂20 g,蒸馏水定容至1000 mL。LB液体培养基:蛋白胨10 g,酵母膏5 g,氯化钠5 g,蒸馏水定容至1000 mL。PDA培养基:马铃薯去皮200 g,葡萄糖20 g,琼脂粉20 g,蒸馏水定容至1000 mL。

1.2 试验方法

1.2.1 拮抗细菌的分离 称取土壤样品10.0 g放入装有90 mL无菌水和玻璃珠的三角瓶中,于摇床上振荡30 min后制得土壤样品悬液,然后按比例制成10⁻²、10⁻³、10⁻⁴样品稀释液。分别吸取100 μL上述处理液于NA培养基上,通过稀释涂布法分离菌株,30 °C培养24 h,然后平板上划线纯化、培养并编号,4 °C存放。

1.2.2 拮抗细菌的筛选 采用平板对峙培养法。将保存的细菌活化培养后,十字交叉等距离(距离病原菌2.5 cm左右)划线至已接种核桃树腐烂病病原菌 *C. chrysosperma* 的PDA平板上^[24],以只接病原菌为对照,每个处理3个重复。26 °C恒温培养,待对照菌丝即将长满时,观察菌落形态并且测量菌落直径计算菌落生长抑菌率。

抑菌率/%=(对照平板菌落直径-处理平板菌落直径)/对照平板菌落直径×100。

1.3 拮抗菌株的鉴定

1.3.1 拮抗菌株形态学特征和生理生化特性测定 将活化后的菌株WS-04划线接种于NA固体培养基上,30 °C培养24 h,观察菌落颜色和形态,并参考《伯杰细菌鉴定手册》^[25]和《常见细菌系统鉴定手册》^[26]测定生理生化指标。

1.3.2 拮抗菌株分子生物学鉴定 拮抗细菌基因组DNA提取采用试剂盒(生工生物科技有限公司),采用细菌通用引物27F5'-AGAGTTTGATCCTGGCT-CAG-3';1492R5'-GGTTACCTTGTACGACTT-3'^[27]扩增16S rDNA;扩增采用50 μL反应体系,PCR反应条件为95 °C预变性3 min,95 °C变性35 s,55 °C退火35 s,72 °C延伸2 min,35个循环,72 °C延伸10 min,扩增产物用1%的琼脂糖凝胶电泳,使用凝胶成像仪观察结果,最后将检测合格的扩增产物送至上海生物工程股份有限公司测序。利用MEGA 11.0软件采用邻接法构建基于16S基因的系统发育树,明确拮抗菌株的分类地位。

1.4 拮抗菌株抑菌谱测定

采用平板对峙法对核桃树腐烂病另一种病原菌 *C. nivea*、苹果腐烂病菌 *V. mali*、香梨腐烂病菌 *V. ambiens*、杏树腐烂病菌 *C. leucostoma*、沙枣腐烂病菌 *C. chrysosperma*、核桃褐斑病菌 *A. alternata*、棉花黄萎病菌 *V. dahliae*、棉花枯萎病菌 *F. oxysporum*开展抑菌率测定,方法参照1.2.2。

1.5 拮抗菌株生长曲线的测定

将拮抗菌株用接种环挑取放入盛有LB液体培养基的250 mL三角瓶中,30 °C 200 r·min⁻¹控温摇床培养,每2 h测量1次OD₆₀₀,培养80 h,记录数据,绘制生长曲线。

1.6 不同浓度梯度发酵滤液对病原菌菌丝抑制率测定

菌悬液的制备:挑取拮抗菌单菌落接种于液体

培养基中,30 °C 200 r·min⁻¹培养2 d,获得菌悬液。

发酵液的制备:取100 μL拮抗菌菌悬液于12 000 r·min⁻¹室温离心2 min,吸取上清液接种于100 mL的液体培养基中,30 °C 200 r·min⁻¹培养2 d后得到发酵液。

发酵滤液的制备:将发酵液4 °C、12 000 r·min⁻¹离心20 min后取上清液,用0.22 μm滤膜过滤器过滤获得发酵滤液,将获得的发酵滤液放置于4 °C冰箱中备用。

将发酵滤液和融化好的PDA培养基混合,分别配置成发酵滤液含量为3%、6%、9%、12%、15%的培养基平板,中央接种一个培养3 d的*C. chrysosperma*菌饼(直径5 mm),以不含发酵滤液的PDA培养基为对照组,每处理3次重复,26 °C培养3 d,采取菌丝生长速率法测定抑菌率。

1.7 拮抗菌株发酵滤液热稳定性测定

将5个装有10 mL发酵滤液的离心管,分别置于55、65、75、85、95 °C温度条件下水浴30 min,将发酵滤液与融化冷却至55 °C的PDA培养基(1:3)混合倒板,待培养基凝固后在平板中央放置病原菌菌饼,以没有进行温度处理的发酵滤液和PDA培养基(1:3)混合为阳性对照,以未加入发酵滤液的PDA培养基为空白对照,每个处理3次重复,26 °C黑暗培养,直至空白对照组病原菌菌丝长满平皿后,测量病原菌菌落直径,并计算菌丝生长抑制率。

1.8 发酵液对*C. chrysosperma*菌丝生长的影响

取20 mL冷却的PDA与5 mL发酵液混合后,在灭菌载玻片上倒上一层混合液,凝固后用灭菌刀片划去宽约1 cm的培养基,挑取培养3 d的*C. chrysosperma*菌丝接种于截面,盖上盖玻片,然后置于湿润的培养皿中26 °C恒温培养3 d,以不加发酵液的PDA为对照。显微镜下观察菌丝形态变化。

1.9 发酵液对离体枝条的保护作用

枝条处理:采集健康和粗细程度均一的2年生核桃枝条,剪成10~15 cm的枝段,在超净工作台用灭菌水冲洗干净后,用0.6%次氯酸钠溶液消毒15~20 min后,无菌水再次清洗3~4次,自然晾干。用水浴锅融化的石蜡封住枝条两端保湿,静置晾干备用,整个过程均在无菌环境进行。

将处理好的枝条用灭菌打孔器(孔径5 mm)打孔,处理组织用灭菌刷子蘸取适量发酵液涂刷枝条5次,等整体晾干后在枝条已打好的孔径处接种核

桃树腐烂病菌菌饼,每个枝条1个接种点,每个处理设置3个重复(每重复为5个枝条),以LB液体培养基代替发酵液为对照,在26 °C条件下保湿培养15 d后,观察离体枝条腐烂病的发生情况,并测量病斑面积(游标卡尺)。

$$\text{病斑面积}/\text{cm}^2 = 1/4 \times \pi \times \text{长径} \times \text{短径}.$$

1.10 菌株WS-04发酵滤液对核桃种子和幼苗的促生作用

1.10.1 菌株WS-04对温185核桃种子萌芽率、发芽率和坏种率的影响选取种仁饱满、无霉无虫,且单果质量≥12 g的干果作为试验材料,自然晾干。将拮抗菌株WS-04发酵滤液加入到灭菌蒸馏水,设置成5个质量浓度(50、150、250、350、450 mg·mL⁻¹),以灭菌蒸馏水处理为对照,一个质量浓度处理20粒种子,3次重复。浸泡之前将核桃外壳开口,使其发酵滤液接触核桃仁,浸泡1 d后用清水洗净,放在湿润的发芽盒内,置于30 °C恒温培养箱中催芽处理,每日观察种子发芽情况,及时清除腐烂霉变种子。待种子发芽后,播种于花盆(20 cm×20 cm)中,每穴2粒种子,覆盖1 cm厚基质(蛭石:珍珠岩:泥炭:土壤体积比为1:1:1:1)。统计种子萌动情况,计算种子萌芽率,待种子长出2枚叶子后统计发芽率和坏种率。

种子萌芽率/%=(催芽后种子露白数/供试种子数)×100;

发芽率/%=(发芽总粒数/供试种子数)×100;

坏种率/%=(种子霉烂数/供试种子数)×100。

1.10.2 菌株WS-04发酵滤液对核桃幼苗的促生作用带壳核桃种子的预处理:将带壳核桃使用核桃夹轻轻裂开,避免核桃仁接触不到拮抗菌发酵滤液。以150 mg·mL⁻¹拮抗菌发酵滤液为试验组,以未添加拮抗菌发酵滤液的培养基为对照,25粒核桃种子为一个处理,5次重复。

浸种催芽处理:核桃种子用75%乙醇表面消毒30 s,无菌水漂洗3次。30 °C温水浸种4~5 h,种子于28 °C催芽。种子发芽后,播种于穴盘(32孔,6 cm×4.5 cm)中,每穴1粒种子,覆盖1 cm厚基质(蛭石:珍珠岩:泥炭:土壤体积比为1:1:1:1)。

促生苗处理:(1)穴盘苗处理。将催芽处理的核桃种子播种于穴盘,开始浇灌拮抗菌发酵滤液(试验组浇灌150 mg·mL⁻¹发酵滤液,对照浇灌水),每次每穴浇灌50 mL、每5 d浇灌一次,待种子生长20 d后,

将穴盘幼苗移栽至室外大田盆栽。

(2)盆栽苗处理。于2023年7月将穴盘盆栽核桃幼苗移栽到塔里木大学节水灌溉试验田(直径为40 cm花盆),浇灌3次发酵滤液每次每盆500 mL(每10 d 1次),核桃幼苗移栽生长90 d后,于2023年9月随机选择5株幼苗测量干质量、苗高、主根长、须根数等生物学指标。拔出核桃幼苗时保证植株的完整性,对核桃幼苗的根系整理时,用无菌水缓慢冲洗,确保根部的完整性。

1.11 数据处理

利用Excel和DPS软件进行数据统计和分析,利用MEGA 11.0建立系统发育树。

2 结果与分析

2.1 拮抗菌株分离和筛选

采用稀释涂布平板法总共分离得到细菌157株,平皿对峙获得5株对*C. chrysosperma*有较强拮抗

作用的菌株,其抑菌率超过70%(表1)。其中菌株WS-04对*C. chrysosperma*抑制作用最强(图1),抑制率达到87.00%。因此本研究中选定菌株WS-04进一步开展后续研究。

2.2 拮抗菌株WS-04的鉴定

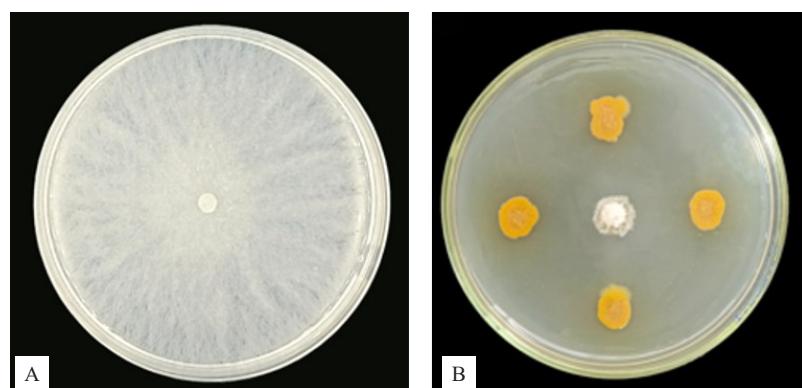
表1 拮抗菌株的复筛结果

Table 1 Rescreening results of the antagonistic strains

菌株编号 Strain number	菌落直径 Colony diameter/cm	抑制率 Antibacterial rate/%
WS-01	2.29±0.18 a	71.67±0.25 c
WS-04	1.07±0.21 c	87.00±0.26 a
WS-10	1.72±0.24 b	78.67±0.32 b
WS-29	2.13±0.21 ab	73.33±0.25 bc
WS-56	2.10±0.35 ab	73.67±0.04 bc

注:表中数据为平均值±标准差,不同小写字母代表差异显著($p<0.05$)。下同。

Note: The data are mean±standard deviation, different small letters represent the significant difference ($p<0.05$). The same below.



A. CK; B. WS-04 菌株对*C. chrysosperma* 的抑菌作用。
A. CK; B. The bacteriostatic effect of strain WS-04 on *C. chrysosperma*.

图1 菌株WS-04对*C. chrysosperma*的抑制作用

Fig. 1 Strain WS-04 confronted the *C. chrysosperma*

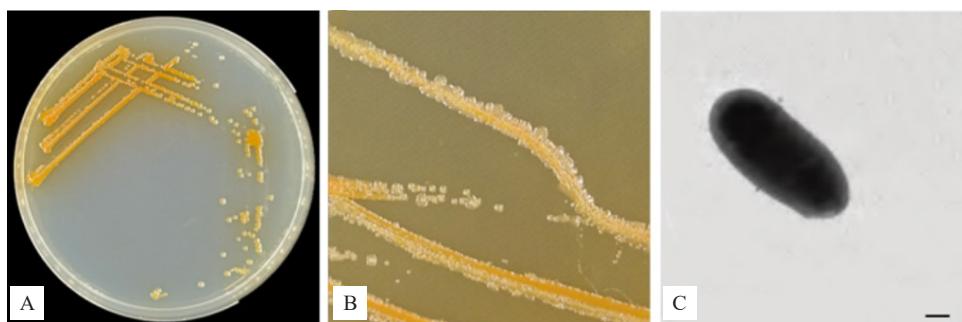
2.2.1 形态特征观察 在LB固体培养基上培养24 h后可形成1.2 mm菌落,菌落橙色,圆形,表面凸起,光滑,较黏稠,易挑起,边缘整齐,电镜扫描菌体形态为杆状(图2)。生理生化测定结果表明,菌株可利用分解无机磷、蛋白酶、铁载体、明胶、多糖、M.R、蔗糖、精氨酸、酯酶、氧化酶、柠檬酸盐。最适生长盐含量(w)为0%~6% NaCl,最适生长pH为7.0~7.5(表2)。

2.2.2 分子生物学鉴定 16S rRNA基因序列测序后提交GenBank获得登录号PP059680,同源性比对结果显示菌株WS-04与绿针假单胞菌*P. chlororaphis*

的同源率为99%。以*Bacillus subtilis*为外群构建系统发育树,菌株WS-04与绿针假单胞菌*P. chlororaphis*聚在同一分支(图3),表明WS-04与*P. chlororaphis*的亲缘关系最近,结合形态特征、生理生化特征和分子鉴定结果,最终将菌株WS-04鉴定为绿针假单胞菌*P. chlororaphis*。

2.3 拮抗菌株WS-04抑菌谱的测定

抑菌谱测定结果表明,拮抗菌株WS-04对8种供试病原菌的抑制率均高于75%(表3)。其中对*C. nivea*、*V. mali*、*V. ambiens*、*V. dahliae*、*C. chrysosperma*、*A. alternata*病原菌的抑制率超过80%,说明



A 和 B 的菌落形态为菌株 WS-04 的培养形态和菌落形态;C. 菌株 WS-04 在扫描电镜下的形态;比例尺=20 μm。
A and B are the culture morphology and colonial morphology of strain WS-04; C. Morphology of strain WS-04 under scanning electron microscopy; Scale bar=20 μm.

图 2 菌株 WS-04 形态特征
Fig. 2 Colony morphology of strain WS-04

表 2 菌株 WS-04 的生理生化鉴定

Table 2 Physiological and biochemical identification of strain WS-04

项目 Item	结果 Result	项目 Item	结果 Result
无机磷 Inorganic phosphorus	+	蔗糖转化果聚糖 Sucrose to fructan	+
有机磷 Organophosphorus	-	精氨酸双水解酶 Arginine dihydrolase	+
纤维素 Cellulose	-	脂酶 Lipoidase	+
蛋白酶 Protease	+	氧化酶 Oxidase	+
淀粉分解 Amyloyisis	-	柠檬酸盐 Citrate	+
几丁质 Chitin	-	脓青素 Pyocyanin	-
铁载体 Ironophore	+	革兰氏染色 Gram stain	-
明胶液化 Gelatin liquefaction	+	NaCl 0%~6%	+
胞外多糖 Exopolysaccharides	+	NaCl 7%	-
M.R	+	pH 7.0~7.5	+

注:“+”. 阳性;“-”. 阴性。

Note: “+”. Positive or available; “-”. Negative or not available.

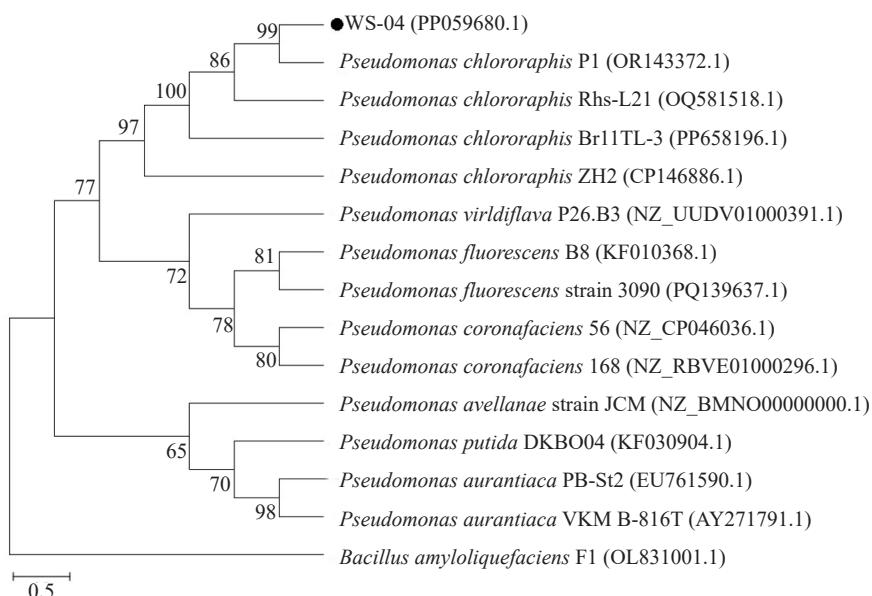


图 3 菌株 WS-04 基于 16S rDNA 基因序列的系统发育树
Fig. 3 Phylogenetic tree of strain WS-04 based on the 16S rDNA gene sequences

表3 菌株 WS-04 对供试病原菌的抑制率

Table 3 Inhibition rate of biocontrol bacteria WS-04 against the tested pathogens

菌株 Strain	抑制率 Inhibition rate/%
<i>C. nivea</i>	82.00±0.02 c
<i>C. leucoostoma</i>	79.10±0.01 c
<i>V. mali</i>	84.07±0.04 b
<i>V. ambiens</i>	87.00±0.01 a
<i>F. oxysporum</i>	78.37±0.01 c
<i>V. dahliae</i>	86.13±0.01 ab
<i>C. chrysosperma</i>	85.21±0.01 ab
<i>A. alternata</i>	84.66±0.04 b

菌株 WS-04 具有较好广谱拮抗效果(图4)。

2.4 拮抗菌株 WS-04 生长曲线的测定

菌株 WS-04 的生长曲线如图5所示,在0~6 h 菌株生长缓慢,进入生长延滞期;6 h 以后细菌快速增长,48 h 达到生长高峰期,随后进入生长稳定期;

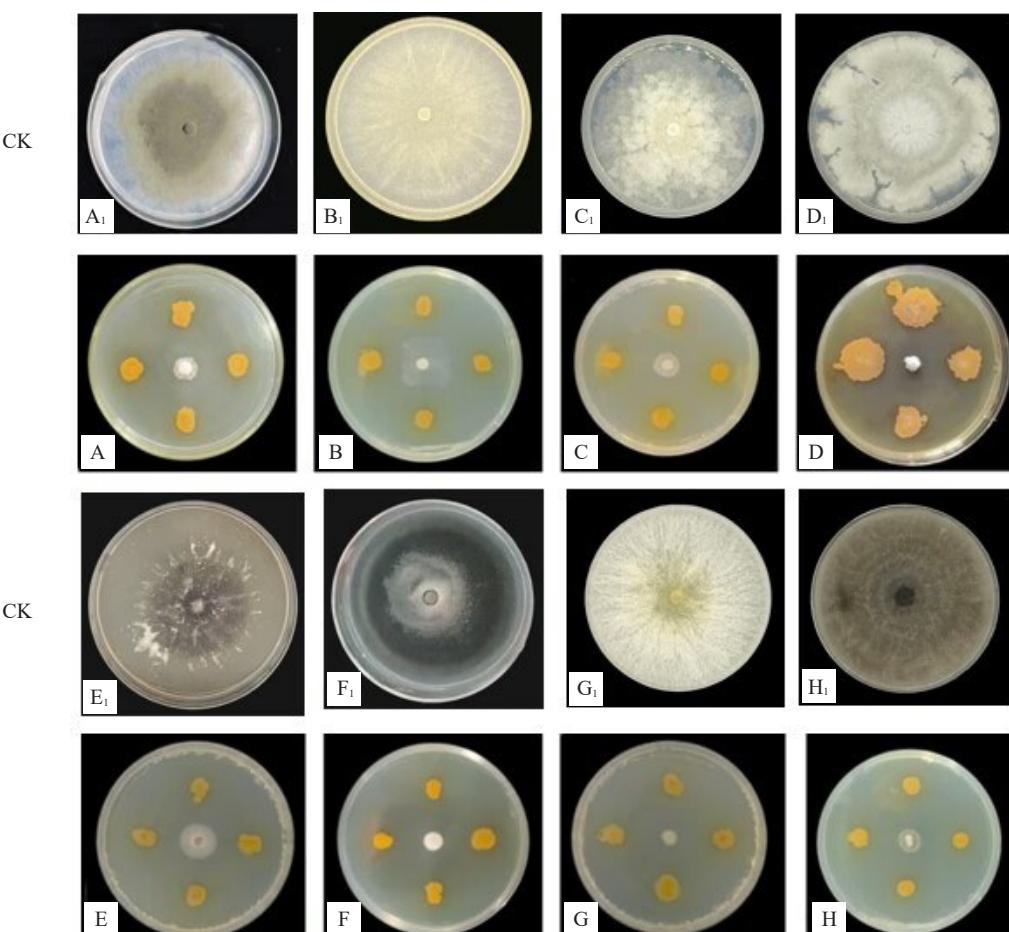
自58 h 以后菌体数量逐渐减少进入衰亡期。研究表明菌株在48~58 h 菌悬液数量最多而且活性强。

2.5 不同浓度梯度发酵滤液对病原菌菌丝的抑制率

采用菌丝生长速率法测定不同浓度梯度发酵滤液对 *C. chrysosperma* 菌丝生长的抑菌率。结果表明不同浓度梯度发酵滤液对菌丝均具有较好抑制效果(图6),且随着浓度升高,其抑制效果越明显,最高抑制率达到93.65%(表4)。

2.6 拮抗菌 WS-04 发酵滤液热稳定性测定

由图7可知,菌株 WS-04 发酵滤液在不同水浴温度处理后,其抑制率随温度升高而下降,但对 *C. chrysosperma* 的抑制率均在85%以上,表现出良好的热稳定性(图7-A、B)。但是95 °C 处理30 min 后,其抑制率明显降低。综上可知,拮抗菌 WS-04 发酵滤液经温度梯度处理以后,对 *C. chrysosperma* 的抑制率有



A₁ 和 A. 核桃树腐烂病病原菌;B₁ 和 B. 杏树腐烂病菌;C₁ 和 C. 苹果树腐烂病菌;D₁ 和 D. 香梨树腐烂病菌;E₁ 和 E. 棉花枯萎病菌;F₁ 和 F. 棉花黄萎病菌;G₁ 和 G. 沙枣树腐烂病菌;H₁ 和 H. 核桃褐斑病菌。

A₁ and A. *C. nivea*; B₁ and B. *C. leucoostoma*; C₁ and C. *C. ambiens*; D₁ and D. *C. ambiens*; E₁ and E. *F. oxysporum*; F₁ and F. *V. dahliae*; G₁ and G. *C. chrysosperma*; H₁ and H. *A. alternata*.

图4 WS-04 对 8 种病原菌菌丝生长的影响

Fig. 4 Effect of WS-04 on mycelial growth of eight plant pathogens

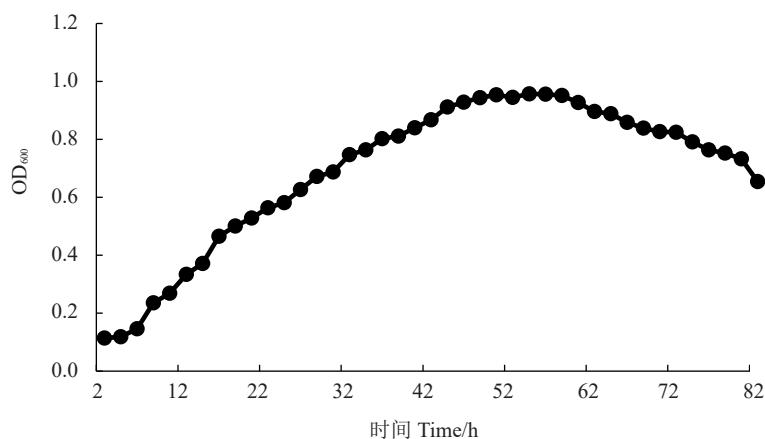


图 5 菌株 WS-04 的生长曲线

Fig. 5 Growth curves of strain WS-04

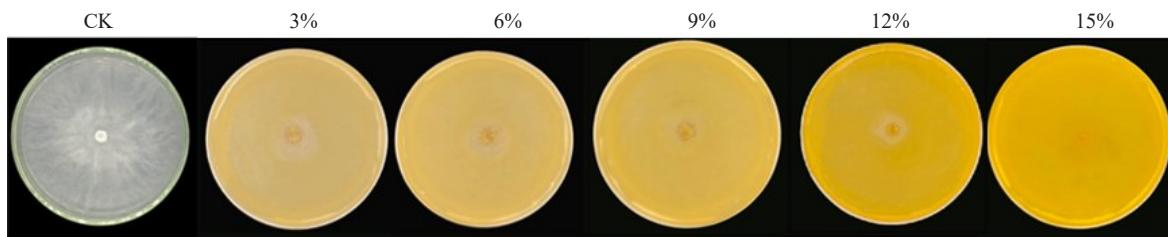
图 6 不同浓度发酵滤液对 *C. chrysosperma* 菌丝生长的抑制效果Fig. 6 Inhibition of fermentation filate growth at different concentrations in *C. chrysosperma*

表 4 不同浓度发酵液对病原菌菌丝生长的抑制率

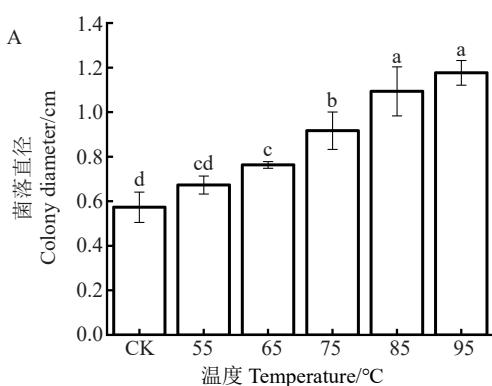
Table 4 Inhibition of hyphal growth of fermentation broth at different concentrations

w	菌落直径 Colony diameter/cm	抑制率 Inhibition rate/%
CK	7.92±0.10 a	0.00
3%	1.44±0.06 b	84.54±4.66 b
6%	1.08±0.18 c	86.32±2.22 b
9%	0.73±0.08 d	90.78±1.03 b
12%	0.56±0.01 de	92.89±0.60 a
15%	0.50±0.01 e	93.65±0.08 a

一定的影响,说明温度升高会导致发酵滤液中抑菌成分逐渐丧失活性。

2.7 拮抗菌 WS-04 发酵液对 *C. chrysosperma* 菌丝生长的影响

由图8可以看出, *C. chrysosperma* 的菌丝在混合培养基上生长3 d后, 菌丝顶端膨大出现皱缩, 局部菌丝弯曲变畸, 菌丝出现严重消融的现象, 菌丝无法正常生长(图8-B、C、E); 而对照组 *C. chrysosperma*



A. 菌株 WS-04 处理后的菌落直径; B. 菌株 WS-04 处理后的抑制率。不同小写字母表示差异显著($p<0.05$)。下同。

A. Colony diameter of strain WS-04 after treatment; B. Inhibition rate of strain WS-04 after treatment. Different small letters indicate significant difference ($p<0.05$). The same below.

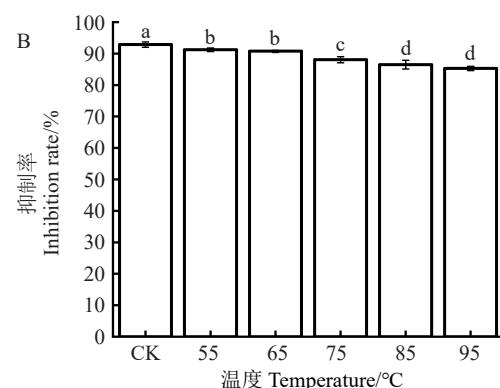
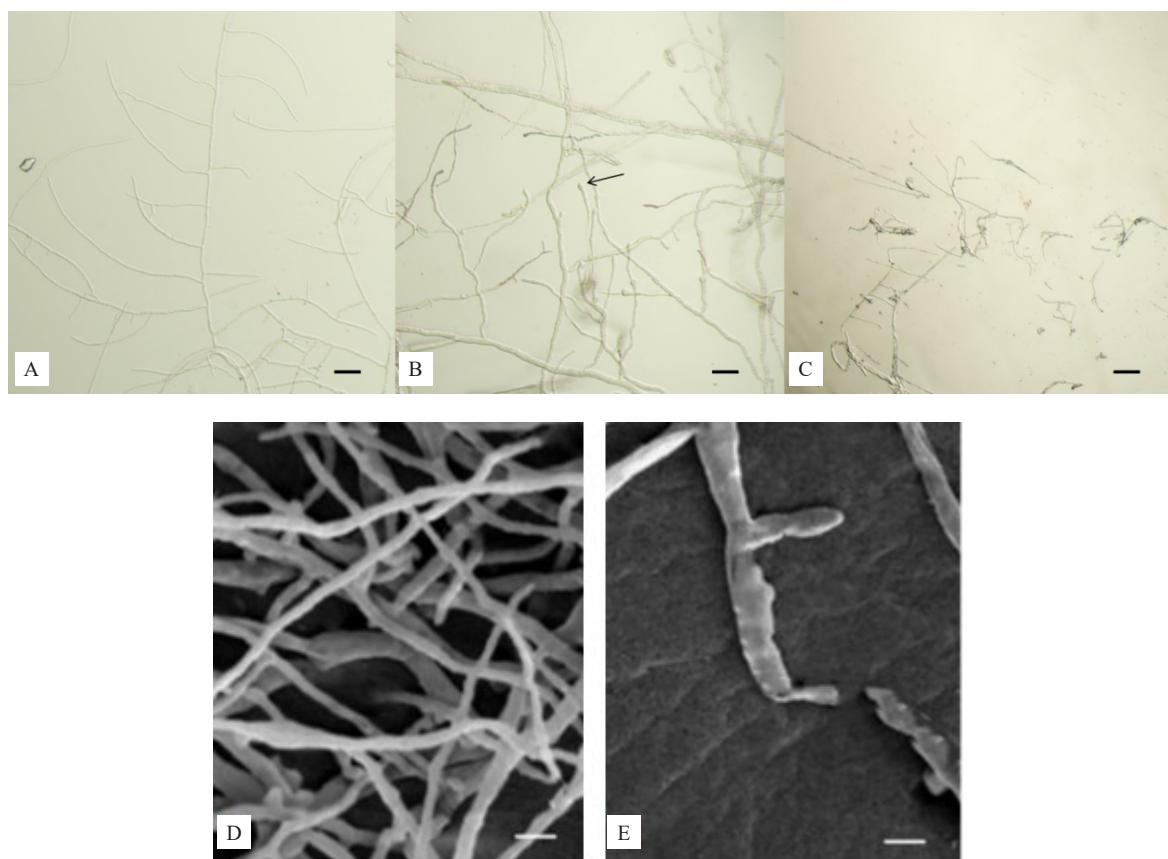


图 7 温度对菌发酵滤液抑菌活性的影响

Fig. 7 Effect of temperature on bacteriostatic activity of biocontrol bacteria fermentation filtrate



A. 正常培养的金黄壳囊孢菌丝;B 和 C. WS-04 处理后的金黄壳囊孢菌丝;D. EM 扫描正常菌丝;E. 扫描 WS-04 处理后的菌丝;箭头:处理后的菌丝膨大变化;标尺=20 μm。

A. *C. chrysosperma* hyphae in normal culture; B and C. WS-04; D. EM scanning normal hyphae; E. Scan WS-04. Arrow: Mycelial enlargement change after treatment; Scale =20 μm.

图 8 绿针假单胞菌 WS-04 对 *C. chrysosperma* 抑制效果

Fig. 8 Inhibition efficacy of *Pseudomonas* WS-04 on *C. chrysosperma*

菌丝细长均匀且表面光滑,形态饱满而完整,能正常生长(图8-A、D)。

2.8 离体枝条保护试验

离体枝条保护试验表明(图9),菌株WS-04对核桃腐烂病具有很好的预防效果,用菌株WS-04发酵液处理过的病斑平均面积为 0.85 cm^2 (图9-B),而对照病斑平均面积达到 6.32 cm^2 (图9-A),防治效果为84.96%(表5)。

2.9 拮抗菌株WS-04发酵滤液对核桃种子和幼苗的促生作用

2.9.1 拮抗菌株WS-04对核桃种子萌芽率、发芽率和坏种率的影响 由图10-A可知,带壳核桃种子在拮抗菌发酵滤液的处理下,随着菌发酵滤液质量浓度的增加,其萌芽率呈现先上升后下降的趋势,而且处理质量浓度在 $150 \text{ mg} \cdot \text{mL}^{-1}$ 和 $250 \text{ mg} \cdot \text{mL}^{-1}$ 左



A. 阳性对照;B. WS-04 对核桃腐烂病的防治效果。

A. Positive control; B. Effect of strain WS-04 on walnut rot spots.

图 9 WS-04 发酵滤液对核桃腐烂病的防治作用

Fig. 9 Control effect of WS-04 fermentation filtrate on walnut canker disease

表 5 WS-04 发酵滤液对核桃腐烂病斑病的防治作用
Table 5 Control effect of WS-04 fermentation filtrate on walnut canker disease

菌株编号 Strain number	病斑面积 Lesion area/cm ²	预防效果 Prevention effect/%
CK	6.32±0.35 a	
WS-04	0.95±0.10 b	84.96

右时能显著提高核桃种子萌芽率。绿针假单胞菌 WS-04 不同质量浓度发酵滤液处理后的种子萌芽情况为 $250 \text{ mg} \cdot \text{mL}^{-1} > 150 \text{ mg} \cdot \text{mL}^{-1} > 350 \text{ mg} \cdot \text{mL}^{-1} > 50 \text{ mg} \cdot \text{mL}^{-1} > 450 \text{ mg} \cdot \text{mL}^{-1}$, 各处理均显著高于对照 ($p < 0.05$)。因此绿针假单胞菌 WS-04 具有促进核桃种子萌芽的作用。

由图 10-B 可知, 带壳核桃种子在拮抗菌不同质量浓度发酵滤液处理下, 发芽率随质量浓度增加呈现先升后降的趋势。绿针假单胞菌 WS-04 发酵滤液在 $50\sim150 \text{ mg} \cdot \text{mL}^{-1}$ 范围内, 核桃种子发芽率逐渐升高, 当发酵滤液质量浓度超过 $250 \text{ mg} \cdot \text{mL}^{-1}$ 时其

发芽率逐渐减弱。拮抗菌发酵滤液质量浓度在 $150\sim250 \text{ mg} \cdot \text{mL}^{-1}$ 之间时, 对核桃种子发芽有着明显促进作用。由图 10-C 可知, 随着发酵滤液质量浓度增加, 核桃种子坏种率呈现出先下降后略微升高的趋势。绿针假单胞菌 WS-04 在质量浓度为 $150 \text{ mg} \cdot \text{mL}^{-1}$ 时的坏种率最低为 6.68%。当拮抗菌发酵滤液质量浓度大于 $150 \text{ mg} \cdot \text{mL}^{-1}$ 以后坏种率呈现上升趋势, 但各质量浓度处理后的坏种率均显著低于对照 ($p < 0.05$)。

2.9.2 菌株 WS-04 发酵滤液对核桃幼苗的促生作用 由表 6 可知, 用绿针假单胞菌 WS-04 处理后的核桃幼苗的平均生长指标(图 11), 其苗高为 16.97 cm 、主根长 18.7 cm 、须根数为 12.33 条、叶片面积为 65.51 cm^2 、干质量为 3.73 g 。而对照组核桃幼苗的苗高为 13.53 cm , 主根长为 10.20 cm , 须根数为 8.33 条, 叶片面积为 52.51 cm^2 , 干质量为 2.33 g 。与对照相比, 其苗高、主根长、须根数、叶片面积和干质量显著增加, 其依次增加了 25.42% 、 83.33% 、 48.02% 、 24.76% 和 98.40% 。

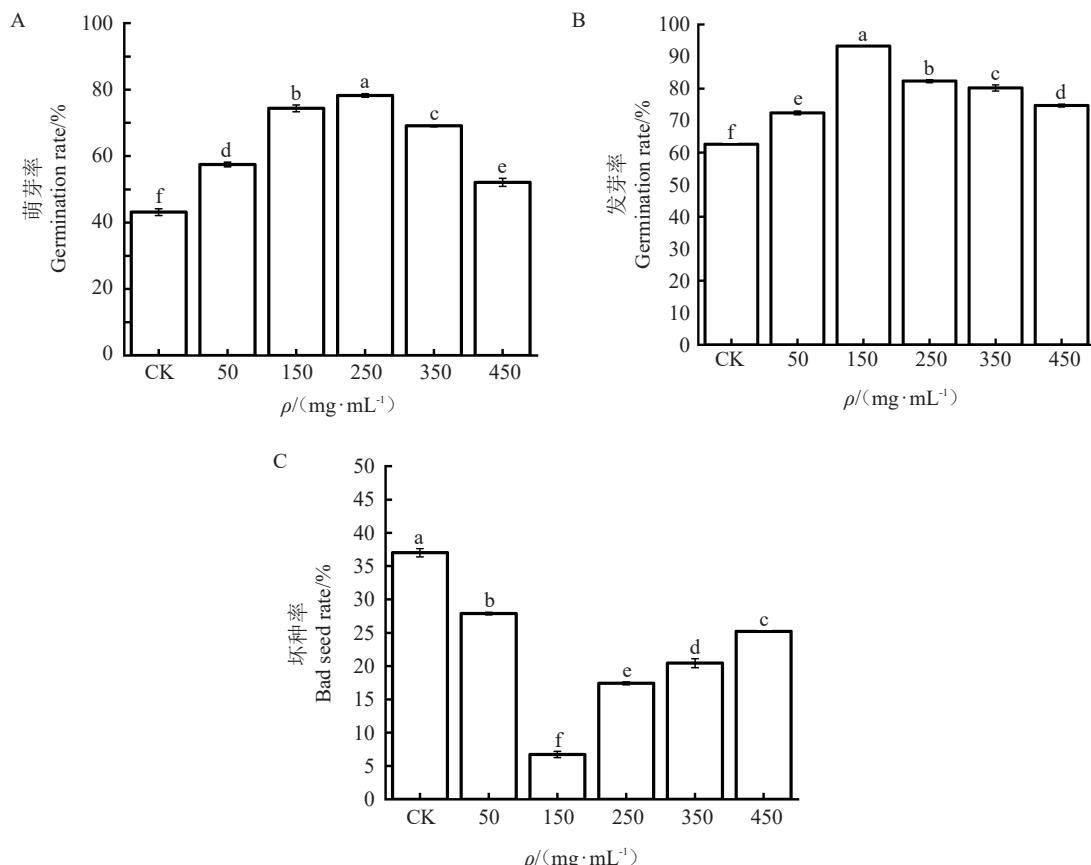


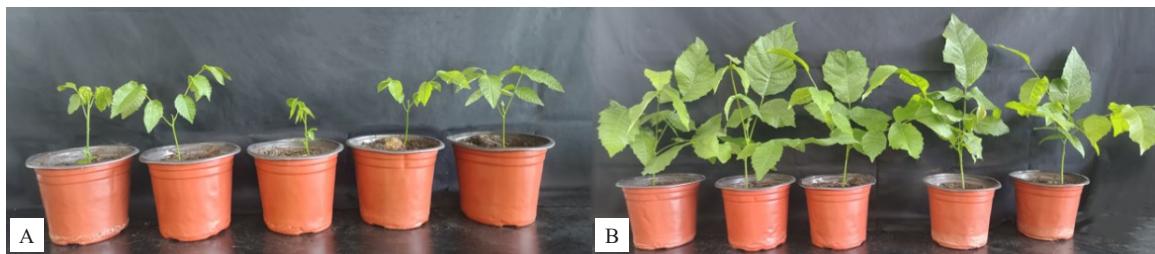
图 10 不同发酵滤液质量浓度对带壳核桃种子萌芽率、发芽率以及坏种率的影响

Fig. 10 The effects of different fermentation filtrate concentrations on the germination rate, germination rate and bad seed rate of shelled walnut seeds

表 6 菌株 WS-04 发酵滤液对核桃幼苗生长指标的影响

Table 6 Effects of fermentation filtrate of strain WS-04 on growth indexes of walnut seedlings

菌株编号 Strain number	苗高 Plant height/cm	主根长 Root length/cm	须根数 Fibrous root number	叶片面积 Blade area/cm ²	干质量 Dry mass/g
CK	13.53±1.12 b	10.20±0.62 b	8.33±1.52 b	52.51±0.73 b	2.33±0.75 b
WS-04	16.97±1.33 a	18.70±13.12 a	12.33±2.08 a	65.51±2.08 a	3.73±0.69 a
增长率 Increase rate/%	25.42	83.33	48.02	24.76	98.40



A. 对照; B. 菌株 WS-04 处理的带壳核桃幼苗。

A. Control; B. Treated walnut seedlings with strain WS-04.

图 11 菌株 WS-04 对核桃幼苗的促生试验

Fig. 11 Growth promotion test of strain WS-04 on walnut seedlings

3 讨 论

从核桃园土壤中分离筛选到一株对核桃腐烂病有较强拮抗作用,同时又能提高核桃萌芽率并降低坏种率的绿针假单胞菌 WS-04,该菌株可使 *C. chrysosperma* 菌丝消融、萎缩、畸变,其抑制率达到 87.00%,核桃离体枝条腐烂病防效亦达到 84.96%。经过绿针假单胞菌 WS-04 发酵滤液处理过的核桃种子,其萌芽率和发芽率明显提高,同时坏种率也明显下降。这是首次报道绿针假单胞菌在核桃上的应用,但绿针假单胞菌的发酵条件、不同环境核桃园根际定殖能力以及防病促生的机制还有待进一步深入研究。

生物防治是实现植物病害绿色防控的有效手段,发掘对病原物具有拮抗作用的有益微生物是实施生物防治的前提^[28]。很多具有拮抗作用的微生物产生的抗菌物质对真菌菌丝形态有明显破坏作用,如 *P. chlororaphis* CY02 处理水稻稻瘟病菌后,导致分生孢子干瘪畸形,菌丝肿胀,隔膜不清晰^[29]。Broadbent 等^[30]发现假单胞杆菌 (*Pseudomonas* spp.) 主要作用机制是菌株产生的抗菌素有溶解烟草黑胫病菌菌丝的功能。假紫色色杆菌的菌株发酵液处理纹枯菌菌丝后,菌丝变形扭曲,萎缩并局部肿胀^[31]。绿针假单胞菌菌株 SPS-41 产生的挥发性气体 3-甲基-1-丁醇、2-甲基-1-丁醇和苯乙醇对甘薯黑斑病病

菌有显著的拮抗作用^[32]。笔者在本研究中从核桃园土壤中分离到一株绿针假单胞菌 WS-04,该菌株能够使 *C. chrysosperma* 菌丝严重畸变甚至消融,表现出了很好的抑菌效果,核桃离体枝条腐烂病的防治效果也非常好,而且还具有一定的热稳定性,该菌株展现出了良好的生物防治前景。绿针假单胞菌 WS-04 发酵滤液经 5 个温度梯度处理 30 min 后,随着温度升高,对 *C. chrysosperma* 的抑制率有所下降,但是抑制率均在 80% 以上,表现出良好的热稳定性。史娜艳从核桃树分离筛选的枯草芽孢杆菌不仅能有效防治真菌病害且还具有热稳定性。说明枯草芽孢杆菌和绿针假单胞菌具有相似的生理特性^[21],但是笔者在本研究中发现假单胞菌会提前产生大量色素限制病原菌菌丝的生长^[21]。李宝燕等^[33]筛选获得一株对果树病害具有较好防治作用的绿针假单胞菌 YT-BTa14,菌株发酵滤液经温度梯度处理表现出较好的热稳定性,这与本研究结论一致。但是绿针假单胞菌与核桃腐烂病菌的作用方式与抑菌机制还不清楚,同时实验室条件下的抑菌试验和接种试验,与大田环境完全不同,绿针假单胞菌能否在大田中发挥其稳定的作用,也需要进一步研究。

一些拮抗菌株在对病原物产生破坏作用的同时,还能产生对植物起到很好促生作用的次生代谢产物。如番茄根际 *P. piscium* 不仅具有抑制尖孢镰刀菌的能力,而且还有较强的溶磷作用,因此能有效

促进盆栽番茄苗的生长^[34]。绿针假单胞菌能够产生吩嗪化合物、铁载体、挥发性物质等,对番茄、玉米、小麦等多种农作物具有促生的功能^[35-37]。本研究中分离到的绿针假单胞菌,其发酵液能够有效促进核桃种子萌芽,同时还能大大降低坏种率,为核桃育好苗、培壮苗提供了优异的生物制剂材料,而且菌株发酵滤液能促进核桃幼苗苗高、主根长、须根数、叶片面积和干质量显著增加,依次增加了25.40%、83.33%、48.02%、24.76%和98.40%。王娟等^[38]从小麦根际筛选的绿针假单胞菌 *P. chlororaphis* HG28-5 在盆栽防病试验中对辣椒疫病具有明显的防治效果。秦娟娟等^[39]研究发现荧光假单孢杆菌 XG32,以卡拉胶和草炭为菌剂,处理组的辣椒苗在株高、根长、鲜质量和干质量等方面都有显著提高。Wu 等^[40]从铁皮石斛中提取的内生细菌铜绿假单胞菌 *P. saponiphila*,具有促进辣椒幼苗生长的功能,以上研究结论与本研究结果一致。但是绿针假单胞菌促进核桃种子萌发的原因是什么?其作用机制如何还有待进一步深入研究。

4 结 论

从新疆阿克苏地区30年树龄核桃园土壤中分离并筛选出一株具有广谱抗菌、热稳定性的生防细菌绿针假单胞菌。通过促生特性试验,表明绿针假单胞菌(*P. chlororaphis*)发酵滤液不仅有效促进核桃种子萌芽和发芽,同时还能促进核桃幼苗的苗高、主根长、须根数、叶片面积和干质量显著增加。

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