阮月红,李宗艳,李银,等.岩生型硬叶兜兰内生固氮细菌的筛选及其生物学特性[J].江西农业大学学报,2025,47(4):961-970.
RUAN Y H,LI Z Y,LI Y,et al.Screening and biological characteristics of endophytic nitrogen-fixing bacteria of lithophytic *Paphiopedilum micranthum*[J].Acta agriculturae universitatis Jiangxiensis,2025,47(4):961-970.

岩生型硬叶兜兰内生固氮细菌的筛选及其生物学特性

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摘要:【目的】内生固氮细菌在提高宿主植物吸收利用氮素及适应性进化方面发挥着重要作用。探究岩生型硬叶兜兰(*Paphiopedilum micranthum*)内生固氮细菌的多样性,了解其生物学特性,为微生物菌剂的开发提供资源。【方法】以岩生型硬叶兜兰为试验材料,采用组织匀浆法分离叶和根中的内生细菌,结合形态学和16S rRNA分子生物学进行分类鉴定,运用无氮培养法筛选固氮细菌,根据固氮能力及其生长量挑选5株固氮细菌,使用Salkowski比色法及平板法分别检测这5株固氮细菌分泌IAA和产铁载体的能力,并采用单因素法对碳源、氮源及无机盐进行培养条件优化。【结果】分离和鉴定结果显示,共从硬叶兜兰的叶和根中分离获得102株内生细菌,分属于4科15属32种,优势菌属为芽孢杆菌属(*Bacillus*),占总分离菌株数的66.67%;固氮细菌初步筛选结果表明,有90株20种内生细菌具有生物固氮能力,占总分离菌株数的88.24%,其中NY-6(*Fictibacillus enclensis*)、NY-21(*Priestia megaterium*)、NY-47(*Peribacillus frigoritolerans*)、NG-6(*Priestia aryabhattai*)和NG-44(*Bacillus mycoides*)菌株的固氮能力及生长量较高,初步判定这5种菌为固氮细菌,并以其中5株为后续试验的供试菌株;促生特性结果显示,5株固氮细菌均具有分泌IAA和产铁载体的能力。其中,NY-6、NG-6和NG-44号菌株的IAA分泌量较多,在不添加色氨酸的情况下分泌量为(6.07±0.01) μg/mL、(9.29±0.03) μg/mL、(10.76±0.04) μg/mL,在添加色氨酸的情况下IAA分泌量达(19.08±0.01) μg/mL、(20.69±0.05) μg/mL、(20.83±0.04) μg/mL,分别是不添加色氨酸时的3.14倍、2.23倍、1.94倍,而产铁载体能力最强的是NY-6号菌株,可溶性指数达6.37±0.40,显著高于其他固氮细菌;单因素试验结果表明,5株固氮细菌的最适无机盐均为$CaCO_3$,而最适碳源和氮源不同,NY-6和NG-44号菌株的最适碳源、氮源分别为可溶性淀粉和蛋白胨,NY-21和NG-6号菌株分别为蔗糖和牛肉膏,NY-47号菌株为蔗糖和蛋白胨。【结论】分离鉴定的内生细菌共有4科15属32种,优势菌属为芽孢杆菌属,其中,*Calidifontibacillus erzurumensis*、*Heyndrickxia ginsengihumi*、*Niallia taxi*、*Neobacillus cucumis*和*Metabacillus idriensis*是首次从兰科植物中分离获得。有20种90株内生细菌具有生物固氮能力,其中,NY-6、NY-21、NY-47、NG-6及NG-44号菌株被初步判定为固氮细菌。5株固氮细菌均具有分泌IAA和产铁载体的能力,对牛肉膏和蛋白胨的利用率较高。NY-6、NG-6和NG-44号菌株的离体促生能力较突出,可用于研究其对兰科植物氮吸收、同化利用的促进机制。

关键词:岩生型硬叶兜兰;内生固氮细菌;分泌IAA能力;产铁载体能力;培养条件优化
中图分类号:Q948.12+2.3　　**文献标志码:**A　　**开放科学(资源服务)标识码(OSID):**
文章编号:1000-2286(2025)04-0961-10
CSTR:32399.14.aauj.2025082

收稿日期:2025-02-27　　**修回日期:**2025-04-25
基金项目:国家自然科学基金项目(32260414)
Project supported by the National Natural Science Foundation of China (32260414)
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Screening and biological characteristics of endophytic nitrogen-fixing bacteria of lithophytic *Paphiopedilum micranthum*

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Abstract: [**Objective**] Endophytic nitrogen-fixing bacteria play a crucial role in enhancing nitrogen absorption and utilization in host plants, as well as in their adaptive evolution. This study aims to explore the functional diversity of endophytic nitrogen-fixing bacteria in lithophytic *Paphiopedilum micranthum*, understand their biological characteristics, and provide resources for the development of microbial agents. [**Method**] This study used the lithophytic *P. micranthum* as the experimental material, using tissue homogenization to isolate endophytic bacteria from leaves and roots. Bacterial identification was performed through morphological and 16S rRNA molecular techniques. Endophytic nitrogen-fixing bacteria were screened using nitrogen-free culture media. Five strains of nitrogen-fixing bacteria were selected based on their nitrogen fixing ability and growth amount, their ability to secrete IAA and produce siderophores was assessed using the Salkowski colorimetric method and plate assays, respectively. The culture conditions for five strains of nitrogen-fixing bacteria were optimized through single-factor experimental design, focusing on carbon sources, nitrogen sources, and inorganic salt. [**Result**] The results of isolation and identification showed that a total of 102 endophytic bacteria were isolated from the leaves and roots of *P. micranthum*, belonging to 15 genera across 4 families, with 32 species. The dominant genus was *Bacillus*, accounting for 66.67% of the total isolated strains. The preliminary screening results of nitrogen-fixing bacteria indicated that 90 strains belonging to 20 species had the ability of biological nitrogen fixation, accounting for 88.24% of the total isolated strains. Among them, the nitrogen-fixing ability and growth amount of NY-6(*Fictibacillus enclensis*), NY-21(*Priestia megaterium*), NY-47(*Peribacillus frigoritolerans*), NG-6(*Priestia aryabhattai*) and NG-44(*Bacillus mycoides*) are relatively high. The 5 species were preliminarily identified as endophytic nitrogen-fixing bacteria, and five representative strains among them were selected as test strains for subsequent experiments.. The plant growth-promoting traits of the strains showed that all five nitrogen-fixing strains were capable of secreting IAA and producing siderophores. Among them, strains NY-6, NG-6, and NG-44 exhibited the high IAA production, reaching (6.07±0.01) μg/mL, (9.29±0.03) μg/mL, (10.76±0.04) μg/mL when 3 strain were cultured in media without tryptophan. IAA production reaching (19.08±0.01) μg/mL, (20.69±0.05) μg/mL, (20.83±0.04) μg/mL when 3 strain were cultured in media with tryptophan, which were 3.14, 2.23 and 1.94 times higher than the strains without tryptophan, respectively. The strain NY-6 exhibited the strongest siderophore production, with a soluble index of 6.37±0.40, which was significantly higher than other nitrogen-fixing bacteria. Single-factor experiments revealed that the optimal inorganic salt for all 5 strains of nitrogen-fixing bacteria was $CaCO_3$, and their preferred carbon and nitrogen sources varied. For strains NY-6 and NG-44, the optimal carbon and nitrogen sources were soluble starch and peptone, strains NY-21 and NG-6 preferred sucrose and beef extract, strain NY-47 preferred sucrose and peptone. [**Conclusion**] 32 species belonging to 15 genera of endophytic bacteria were identified from 102 strains obtained from leaves and roots of *P. micranthum*. Among them, *Calidifontibacillus erzurumensis*, *Heyndrickxia ginsengihumi*, *Niallia taxi*, *Neobacilluscucumis*, and *Metabacillus idriensis* were isolated from orchid plantsfor the first time. A total of 20 species (90 strains) of endophytic bacteria were found topossess biological nitrogen-fixing capabilities. Among these, strains NY-6, NY-21, NY-47, NG-6, and NG-44 were preliminarily identified as nitrogen-fixing bacteria. All five strains of nitrogen-fixing bacteria have the ability to secrete IAA and siderophore production. Five strains exhibiting high utilization efficiency for beef extract and peptone. Strains NY-6, NG-6 and NG-44 exhibit notable plant growth-promoting abilities in vitro and could be used to investigate their promoting mechanisms in enhancing nitrogen absorption and assimilation in orchids.

Keywords: lithophytic *Paphiopedilum micranthum*; endophytic nitrogen-fixing bacteria; IAA secretion ability; siderophore production ability; culture condition optimization

【研究意义】岩生型硬叶兜兰(*Paphiopedilum micranthum*)生长于喀斯特石灰岩地区的石壁上或石窝的风积土处[1],与生长于灌丛间的地生型硬叶兜兰相比土层浅薄,需依赖根系沿着岩石结构面隙吸取下层土壤中的氮营养[2-3],经课题组前期研究发现,相同生境下岩生型硬叶兜兰比地生型氮利用效率(nitrogen use efficiency,NUE)更高[4]。硬叶兜兰以黔西南、桂北、滇东南的喀斯特石灰岩地区为生境[5],该地区易发生土壤氮养分流失,导致植物生长受限[6],那么,岩生型硬叶兜兰是如何在氮受限的生境中保持较高的氮利用效率呢?植物为了适应复杂多变的环境,往往会通过与微生物共生来促进自身的生长发育[7]。硬叶兜兰属兰科(Orchidaceae)兜兰属(*Paphiopedilum*)[8],是典型的菌异养植物。兰科植物在依赖菌异养提供营养物质的同时,这些菌也决定着兰科植物的氮富集程度[9-10]。目前对于硬叶兜兰共生菌与氮营养关系的研究大多集中在菌根真菌和根际微生物群落结构方面[4,11],内生固氮细菌具有生物固氮能力,能有效促进宿主植物吸收氮营养,筛选岩生型硬叶兜兰内生固氮细菌并测定其生物学特性能进一步了解硬叶兜兰的适应性机制,挖掘对氮代谢具有促进作用的内生细菌资源。【前人研究进展】内生固氮细菌能将大气中的氮转化为氨和其他有机氮化合物,可通过提高宿主植物的氮可用性,进而减少其对合成肥料的依赖,是一种潜在的可持续农业资源[12-13]。农倩等[14]研究表明,接种了内生固氮细菌的甘蔗根、茎和叶从空气中获取的氮分别为7.69%、15.64%和8.72%。固氮细菌通常还具有多种促生特性,如:分泌吲哚-3-乙酸(indole-3-acetic acid,IAA)、产铁载体、解磷和解钾等,张芳芳[15]从五唇兰根部分离获得3株同时具有分泌IAA、解磷和产铁载体能力的内生固氮细菌;赵鹏菲[16]从小沼兰中分离获得2株同时具有分泌IAA、解磷、解钾和产铁载体能力的内生固氮细菌。

【本研究切入点】目前对岩生型硬叶兜兰的内生固氮细菌资源尚不清楚,其生物学特性的研究也极少。【拟解决的关键问题】研究以岩生型硬叶兜兰的叶和根为试验材料,分离、鉴定内生细菌,筛选具有生物固氮能力的内生细菌,并根据固氮能力和生长量挑选5株固氮细菌进行分泌IAA、产铁载体能力的测定和培养条件优化,探究岩生型硬叶兜兰可培养内生固氮细菌资源,了解其生物学特性,为进一步研究内生固氮细菌对兰科植物氮代谢的促进机制提供参考依据。

1　材料与方法

1.1　植物材料及供试培养基

硬叶兜兰采集自滇东南石灰岩地区生长在石壁上或石窝中的植株,采样位置为(23°22′776″N,104°23′041″E)。该区域属于中亚热带季风气候,年均温14.9~16.6 ℃,年均降雨量967~1 870 mm。

内生细菌分离采用NA、YG和KB培养基,纯化采用NA-1培养基,固氮菌筛选采用Ashby无氮培养基,铁载体能力测定采用CAS培养基,培养条件优化采用NP培养基。

1.2　研究方法

1.2.1　硬叶兜兰内生细菌的分离和纯化

内生细菌的分离采用传统的组织匀浆分离法[17]。取叶和根清洗干净,用滤纸擦干水分后,先用75%酒精洗涤1 min,再用0.1%升汞溶液分别漂洗2,3,5 min(根漂洗5,6,7 min),无菌水清洗3次,滤纸擦干水分后,添加适量无菌水研磨成匀浆,吸取100 μL匀浆分别涂布于NA、YG、KB固体培养基上,每个处理重复3次,以最后一次无菌水冲洗液为空白对照,28 ℃暗培养3~5 d,待有菌落长出后,反复纯化直至得到能稳定生长的细菌单菌落。

1.2.2　分离菌株的形态学及分子生物学鉴定

形态学鉴定参考《常见细菌系统鉴定手册》[18]。分子生物学鉴定采用16S rRNA基因测序法[19]。先用Ezup柱式细菌基因组DNA抽提试剂盒(生工生物工程上海股份有限公司)提取DNA,然后按照2×San *Taq* PCR Mix产品说明书将DNA样品添加至PCR反应体系中,在基因扩增仪上进行PCR扩增。PCR反

应体系的上游引物序列为5′-GGTTACCTTGTTACGACTT-3′(1492R),下游引物序列为5′-AGAGTTTGATCMTGGCTCAG-3′(27F)。PCR反应体系:2×San *Taq* PCR Mix 12.5 μL,正、反向引物各1 μL,DNA模板1 μL,ddH_2O 9.5 μL。PCR反应条件:94 ℃预变性5 min;94 ℃变性1 min,56 ℃退火1 min,72 ℃延伸2 min,30个循环;72 ℃再延伸10 min。取5 μL的PCR产物用10 g/L琼脂糖凝胶电泳检测,将扩增的PCR原液送至生工生物工程上海股份有限公司进行双向测序。

1.2.3 内生固氮细菌的筛选

采用无氮培养法初步筛选固氮细菌[20]。将菌株接种至Ashby固体培养基上,28 ℃暗培养7 d,观察菌株是否能正常生长。将能正常生长的菌株在Ashby培养基上连续转接3次,观察菌株的生长状况。

1.2.4 分离菌株分泌IAA能力测定

使用Salkowski比色法对固氮细菌进行定性检测[21]。将菌株分别接种在不添加和添加500 mg/L色氨酸的NA-1培养基中,28 ℃、200 r/min培养72 h,取发酵菌液10 000 r/min离心10 min,取离心后的上清液3 mL于比色容器中,加入等体积的IAA显色剂,充分混合均匀后,放置室温下避光显色30 min,若有红色出现,则说明该菌株具有分泌IAA的能力。使用紫外分光光度计定量检测显色溶液在530 nm处的吸光度(OD_{530})值,每个处理设置3组重复,以空白对照组作为阴性对照进行调零。

1.2.5 分离菌株产铁载体能力测定

采用平板法检测固氮细菌的产铁载体能力[22]。使用5 mm打孔器将单菌落整个挑取接种于CAS固体培养基上,每个菌株3次重复,28 ℃暗培养3~5 d,观察菌饼周围是否有橙色晕圈产生,并采用“十字交叉法”测定晕圈的直径(D)和菌落直径(d),计算其可溶性指数(D/d)。

1.2.6 培养条件优化

以NP培养基为基础培养基,分别用不同碳源(葡萄糖、蔗糖、甘露醇、可溶性淀粉、肌醇和山梨醇)、氮源(牛肉膏、蛋白胨、$(NH_4)_2SO_4$、尿素、胰蛋白胨和KNO_3)和无机盐(NaCl、KH_2PO_4、Na_2HPO_4、$MnSO_4$、$CaCO_3$和柠檬酸钠)替代培养基中的碳源、氮源和无机盐。种子液添加量1%,28 ℃,200 r/min培养24 h,测定菌液的OD_{600}值,试验重复3次,筛选出5株细菌的最适碳源、氮源和无机盐。

1.3 数据处理

数据绘图使用Origin 2021软件,试验数据以3个重复次数的平均值±标准差(mean±SD)表示;数据分析使用Excel 2021和SPSS 27,采用单因素方差(one-way ANOVA)分析检验数据差异显著性($P<0.05$)。

2 结果与分析

2.1 硬叶兜兰内生细菌的分离与鉴定

形态和分子生物学鉴定结果见表1(以表中菌株号对应的细菌种类为代表菌株),本次共从硬叶兜兰的叶和根中分离获得32种102株内生细菌,均属于厚壁菌门(*Firmicutes*)和芽孢杆菌纲(*Bacilli*),分属于4科15属,优势菌属为芽孢杆菌属(66.67%)。从分离的部位来看,根中分离获得的最多,有22种,叶中分离获得17种,根和叶中均能分离获得的有7种。从消毒时间来看(图1A),叶最佳的消毒时间为2 min,分离出15种,根最佳的是6 min,分离出12种(图1B);从分离培养基来看(图1C),NA培养基分离获得的细菌种类最多,有20种(62.5%),其次是KB培养基。

2.2 固氮细菌的筛选

共筛选到90株细菌具有固氮能力(表2),占总数的88.24%,分属于7属20种,芽孢杆菌属为优势菌属,有12种12株不具备固氮能力。经过3次连续接种后发现,具有稳定固氮能力的有40株,分属于6种,叶中分离获得4种24株,根中分离获得5种16株,叶和根中均能分离获得的有3种,分别是NY-2、NY-21和NY-47号菌株,具有强固氮能力的有12种43株,具有固氮能力的有2种7株。其中,NY-6、NY-21、NY-47、NG-6和NG-44号菌株的固氮能力和生长量较高,初步判定这5种内生细菌为固氮细菌,并以其中5株为后续试验的供试菌株。

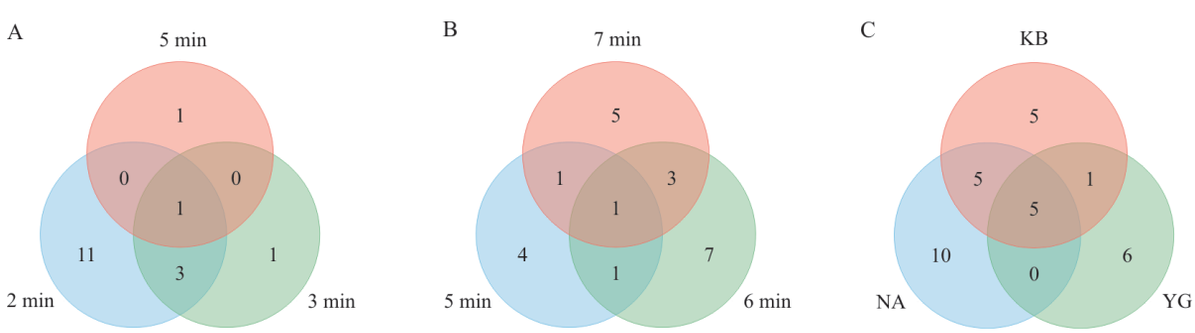
表 1　分离菌株的相似菌株及 GenBank 登录号

Tab.1　Similar strain and GenBank accession No. of the isolated strains

菌株号 Strain number	相似菌株及 GenBank 登录号 Similar strain and GenBank accession No.	菌株号 Strain number	相似菌株及 GenBank 登录号 Similar strain and GenBank accession No.
NY-1	*Bacillus velezensis* CR-502(OQ876683.1)	NY-11	*Calidifontibacillus erzurumensis* P2(NR_178988.1)
NY-2	*Bacillus subtilis* JCM 1465(ON041099.1)	NY-21	*Priestia megaterium* NBRC 15308 (T) (MK424276.1)
NY-7	*Bacillus tequilensis* KCTC13622(MZ573379.1)	NG-6	*Priestia aryabhattai* B8W2(NR_115953.1)
NY-9	*Bacillus ferrooxidans* YT-3(KY628809.1)	NY-30	*Heyndrickxia ginsengihumi* Gsoil 114(MW019944.1)
NY-31	*Bacillus toyonensis* BCT-7112(OQ626001.1)	NY-34	*Niallia taxi* M5HDSG1-1(OL880528.1)
NY-37	*Bacillus stratosphericus* 41KF2a(ON878108.1)	NY-47	*Peribacillus frigoritolerans* DSM 8801(PP757982.1)
NY-54	*Bacillus proteolyticus* TD42(PP757996.1)	NG-2	*Peribacillus muralis* LZ15-41(MT856238.1)
NY-68	*Bacillus paramobilis* BML-BC017 (PP758243.1)	NG-9	*Lysinibacillus composti* NCCP-36(NR_126171.1)
NG-10	*Bacillus mangrovi* JBRI-MO-0023(MK302238.1)	NG-17	*Neobacillus cucumis* V6148(PP257562.1)
NG-11	*Bacillus sonorensis* T-2(OQ750673.1)	NG-22	*Psychrobacillus psychrodurans* NB-9(KU254665.1)
NG-19	*Bacillus kochii* Uyi_40(MT507233.1)	NG-37	*Metabacillus idriensis* 24532(OR430309.1)
NG-31	*Bacillus licheniformis* ATCC 14580(ON795919.1)	NY-24	*Cohnella lubricantis* KSS-154-50(NR_156082.1)
NG-39	*Bacillus simplex* Qtx-11(GU201860.1)	NG-29	*Paenibacillus guangzhouensis* SK2(OM049335.1)
NG-44	*Bacillus mycoides* ATCC 6462(NR_115993.1)	NG-32	*Paenibacillus uliginis* LMITABS02887(OR304339.1)
NG-49	*Bacillus paralicheniformis* FJAT-47814(MG651217.1)	NY-28	*Staphylococcus pasteuri* ATCC 51129(NR_114435.1)
NY-6	*Fictibacillus enclensis* NIO-1003(NR_133744.1)	NG-63	*Sporosarcina koreensis* 22260(OR431528.1)

以“NY-”命名的菌株是从硬叶兜兰的叶中分离出来的;以“NG-”命名的菌株是从硬叶兜兰根中分离获得的。

Strains labeled as “NY-” were isolated from the leaves of *P. micranthum*; while strains labeled as “NG-” were isolated from the roots of *P. micranthum*.



A 为叶消毒时间所分离获得的菌株种数;B 为根消毒时间所分离获得的菌株种数;C 为 NA、YG 及 KB 3 种用以内生细菌分离的培养基分离获得的菌株种数。

A shows the number of strains isolated from leaf disinfection time; B shows the number of strains isolated from root disinfection time; C shows the number of strains obtained by separating NA, YG and KB with culture medium for endophytic bacterial isolation.

图 1　不同消毒时间和不同培养基分离获得的菌株种数

Fig.1　The number of strains isolated under different disinfection times and different culture media

2.3　5 株固氮细菌分泌 IAA 的能力

5 株固氮细菌在不添加色氨酸和添加色氨酸的情况下均产生了红色变化(图 2A)。5 株固氮细菌分泌 IAA 的浓度如图 2B 所示,在不添加色氨酸的情况下 5 株固氮细菌分泌 IAA 的量为 6.07~10.76 μg/mL,而在添加色氨酸的情况下,分泌量为 11.92~20.83 μg/mL。其中,NY-6、NG-6 和 NG-44 号菌株的 IAA 分泌量较多,在添加色氨酸的情况下 IAA 分泌量达(19.08±0.01) μg/mL,(20.69±0.05) μg/mL,(20.83±0.04) μg/mL,分别是不添加色氨酸时的 3.14 倍、2.23 倍、1.94 倍。

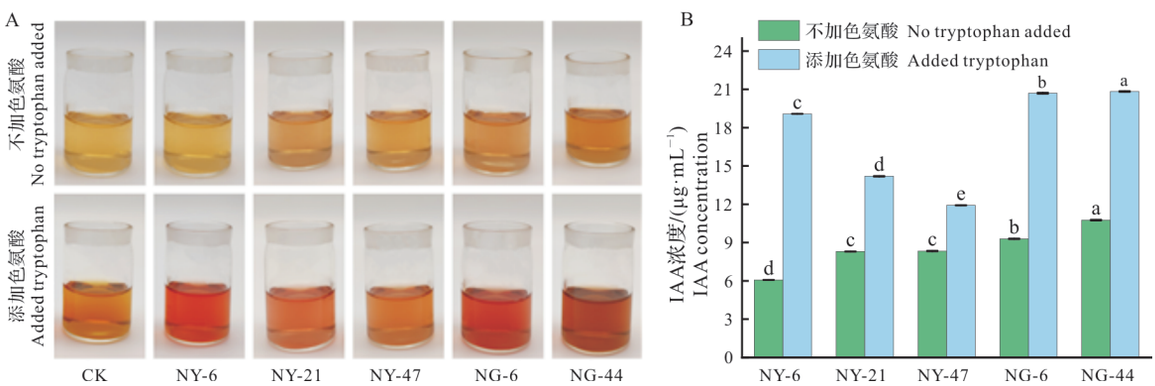
表 2 硬叶兜兰内生细菌固氮能力的差异

Tab.2 Differences in nitrogen-fixing ability of endophytic bacteria in *P. micranthum*

菌株号 Strain number	固氮作用 Nitrogen fixation	菌株号 Strain number	固氮作用 Nitrogen fixation	菌株号 Strain number	固氮作用 Nitrogen fixation	菌株号 Strain number	固氮作用 Nitrogen fixation
NY-1	++	NY-28	−	NG-2	++	NG-29	−
NY-2	+++	NY-30	−	NG-6	++	NG-31	−
NY-6	+++	NY-31	+	NG-9	−	NG-32	++
NY-7	++	NY-34	−	NG-10	−	NG-37	+++
NY-9	−	NY-37	++	NG-11	++	NG-39	+++
NY-11	++	NY-47	+++	NG-17	−	NG-44	++
NY-21	+++	NY-54	++	NG-19	++	NG-49	++
NY-24	−	NY-68	+	NG-22	−	NG-63	−

“+++”:单菌落直径为2~3 mm,具有稳定固氮能力;“++”:单菌落直径为1~2 mm,具有强固氮能力;“+”:单菌落直径小于1 mm,具有固氮能力;“−”:无固氮能力。

“+++”:The diameter of a single colony is 2−3 mm., exhibiting stable nitrogen-fixing ability;“++”:The diameter of a single colony is 1−2 mm., exhibiting strong nitrogen-fixing ability;“+”:The diameter of a single colony is less than 1mm, exhibiting nitrogen-fixing ability;“−”:No nitrogen-fixing ability.



A为5株固氮细菌分泌IAA的效果;B为5株固氮细菌分泌IAA的浓度。

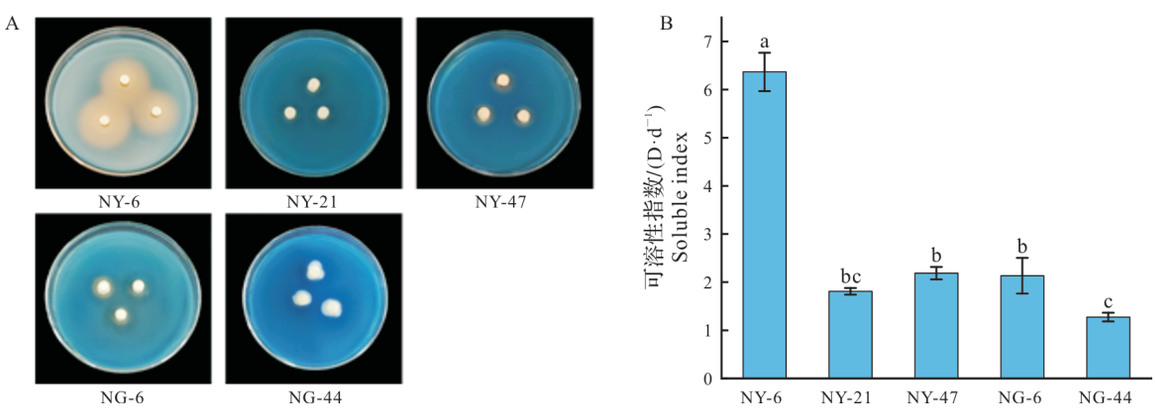
A shows the effect of IAA secretion by 5 strains of nitrogen-fixing bacteria;B shows the concentration of IAA secretion by 5 strains of nitrogen-fixing bacteria.

图2 5株固氮细菌在不添加色氨酸和添加色氨酸条件下分泌IAA的效果和浓度

Fig.2 Effect and concentration of IAA secretion by 5 strains of nitrogen-fixing bacteria under with and without tryptophan

2.4 5株固氮细菌产铁载体的能力

5株固氮细菌均能产生橙色晕圈(图3A),其合成铁载体的可溶性指数如图3B所示,产铁载体能力由强至弱分别是NY-6、NY-47、NG-6、NY-21和NG-44,NY-6号菌株的菌落直径最小,橙色晕圈最大,其可溶性指数最大,为6.37±0.40。



A为5株固氮细菌产铁载体的效果;B为5株固氮细菌产铁载体的可溶性指数。

A shows the effect of siderophore production by 5 strains of nitrogen-fixing bacteria;B shows the soluble index of siderophore production by 5 strains of nitrogen-fixing bacteria.

图3 5株固氮细菌产铁载体的效果及可溶性指数

Fig.3 Effect and soluble index of siderophore production by 5 strains of nitrogen-fixing bacteria

2.5　培养条件优化

2.5.1　不同碳源对5株固氮细菌生长量的影响

NY-6和NG-44号菌株的最适碳源均为可溶性淀粉(表3),其生长量(OD_{600}值)分别可达到(2.61±0)和(2.76±0.02);而菌株NY-21、NY-47和NG-6的最适碳源则均为蔗糖,其生长量(OD_{600}值)分别为(2.58±0.01)、(1.12±0.02)、(3.05±0.01)。

表3　5株固氮细菌在不同碳源下的生长差异

Tab.3　Growth differences of 5 strains of nitrogen-fixing bacteria under different C sources

供试菌株 Strains	葡萄糖 Glucose	蔗糖 Sucrose	甘露醇 Mannitol	可溶性淀粉 Soluble starch	肌醇 Inositol	山梨醇 Sorbitol
NY-6	2.02±0^{f}	2.24±0.01^{e}	2.28±0.01^{d}	2.61±0^{a}	2.51±0.01^{b}	2.41±0^{c}
NY-21	2.42±0^{d}	2.58±0.01^{a}	2.57±0.01^{ab}	2.24±0.01^{e}	2.44±0.01^{c}	2.56±0.01^{b}
NY-47	0.74±0.02^{d}	1.12±0.02^{a}	0.53±0.01^{e}	0.98±0.04^{b}	0.83±0.01^{c}	0.99±0.02^{b}
NG-6	3.03±0.01^{a}	3.05±0.01^{a}	2.66±0^{c}	3.04±0.02^{a}	2.82±0.01^{b}	2.47±0.01^{d}
NG-44	2.70±0.03^{b}	2.26±0.01^{c}	2.27±0.01^{c}	2.76±0.02^{a}	2.27±0.02^{c}	2.25±0.01^{c}

同一行不同字母表示差异显著(P<0.05)。

Different letters on the same line indicate significant differences(P<0.05).

2.5.2　不同氮源对5株固氮细菌生长量的影响

NY-6、NY-47和NG-44号菌株的最佳氮源均为蛋白胨(表4),其中NG-44号菌株的OD_{600}值最大,为3.19±0.01,而NY-21和NG-6的最佳氮源为牛肉膏,OD_{600}值分别是3.15±0.02和3.87±0.03。

表4　5株固氮细菌在不同氮源下的生长差异

Tab.4　Growth differences of 5 strains of nitrogen-fixing bacteria under different N sources

供试菌株 Strains	牛肉膏 Beef Extract	蛋白胨 Peptone	硫酸铵 $(NH_4)_2SO_4$	尿素 Urea	胰蛋白胨 Tryptone	硝酸钾 KNO_3
NY-6	1.47±0.01^{b}	2.2±0^{a}	0.1±0^{e}	0.14±0^{d}	1.33±0.02^{c}	0.09±0^{e}
NY-21	3.15±0.02^{a}	2.13±0.01^{c}	0.08±0.01^{f}	0.35±0^{d}	2.34±0^{b}	0.29±0^{e}
NY-47	0.37±0^{b}	0.67±0.01^{a}	0.05±0^{f}	0.07±0^{e}	0.34±0^{c}	0.09±0^{d}
NG-6	3.87±0.03^{a}	3.42±0.12^{b}	0.07±0^{f}	0.74±0^{d}	2.48±0.01^{c}	0.28±0^{e}
NG-44	3.05±0.02^{b}	3.19±0.01^{a}	0.24±0^{e}	0.32±0^{d}	2.41±0.01^{c}	0.13±0^{f}

同一行不同字母表示差异显著(P<0.05)。

Different letters on the same line indicate significant differences(P<0.05).

2.5.3　不同无机盐对5株固氮细菌生长量的影响

5株细菌的最佳无机盐均为$CaCO_3$(表5),其暗培养24 h后的生长量(OD_{600}值)由大到小依次为NY-21、NG-6、NG-44、NY-6、NY-47,分别是(3.64±0.01)、(3.56±0.02)、(3.30±0.17)、(2.63±0.02)、(2.48±0.02),NY-21号菌株的生长量最大。

表5　5株固氮细菌在不同无机盐组分下的生长差异

Tab.5　Growth differences of 5 strains of nitrogen-fixing bacteria under different inorganic salt components

供试菌株 Strains	氯化钠 NaCl	磷酸二氢钾 KH_2PO_4	磷酸氢二钠 Na_2HPO_4	硫酸锰 $MnSO_4$	碳酸钙 $CaCO_3$	柠檬酸钠 $C_6H_5Na_3O_7$
NY-6	2.26±0.01^{b}	2.05±0.01^{c}	1.92±0.02^{d}	0.21±0^{e}	2.63±0.02^{a}	0.10±0^{f}
NY-21	2.62±0.01^{c}	2.41±0.01^{e}	2.47±0^{d}	0.18±0.01^{f}	3.64±0.01^{a}	2.66±0.01^{b}
NY-47	0.73±0^{b}	0.19±0.01^{c}	0.18±0^{cd}	0.14±0^{e}	2.48±0.02^{a}	0.15±0de
NG-6	2.66±0.01^{c}	2.70±0^{b}	2.70±0^{b}	0.14±0^{e}	3.56±0.02^{a}	2.53±0^{d}
NG-44	2.61±0.01^{b}	2.39±0^{c}	2.62±0.01^{b}	0.11±0^{d}	3.3±0.17^{a}	2.65±0^{b}

同一行不同字母表示差异显著(P<0.05)。

Different letters on the same line indicate significant differences(P<0.05).

3 讨论与结论

植物对环境的适应性可视为宿主植物及其微生物群组成的全生物的适应性[23]，硬叶兜兰对喀斯特氮养分有限的生境具有很强的适应能力[24]，因此研究硬叶兜兰内生固氮细菌的生物学特性可在一定程度上了解内生细菌对宿主植物适应少氮生境的促进机制。

本研究共分离获得15个属，优势菌属为芽孢杆菌属（66.67%），也是铁皮石斛、霍山石斛、金钗石斛和细茎石斛中的优势菌属[25-28]，芽孢杆菌属生长速度快，具有多种促生特性[29]，可作生防剂、生物修复剂及微生物肥料等应用于可持续农业生产。有32种内生细菌，其中 *Calidifontibacillus erzurumensis*、*Heyndrickxia ginsengihumi*、*Niallia taxi*、*Neobacillus cucumis* 和 *Metabacillus idriensis* 是首次从兰科植物中分离获得。

本研究分离的菌株中有20种90株具有固氮能力，占总数的88.24%，表明固氮细菌可能在硬叶兜兰的细菌群落中占据了重要地位。7种芽孢杆菌（*B. toyonensis*、*B. mycoides*、*B. paralicheniformis*、*B. velezensis*、*B. licheniformis*、*B. subtilis*、*B. tequilensis*）和1种 *Peribacillus frigoritolerans* 与多个研究的鉴定结果一致，均具有生物固氮作用[30-32]。白洁等[31]对欧李固氮细菌的筛选结果表明菌株 *B. paralicheniformis* 的固氮酶活性为424.81 nmol/（h·mL），*B. velezensis* 的固氮酶活性为200~300 nmol/（h·mL）。本研究初步筛选的具有固氮能力的细菌还有待进一步测定其固氮酶活性。Li[33]研究表明IAA能通过影响硝酸盐转运蛋白NRT1和NRT2 2个家族的基因表达及植株根系构型（root system architecture，RSA）进而影响植物的NUE，这些具有IAA分泌能力的内生细菌可能在岩生型硬叶兜兰根构型对环境适应性方面发挥着重要作用，本研究所测定的5株固氮细菌均具有分泌IAA的能力，添加色氨酸能促进菌株的IAA合成，说明这些菌株的IAA合成途径可能是以色氨酸依赖型为主。O'hara等[34]在研究缺铁花生对固氮能力的影响时表明，铁可作为辅助因子促进氮的同化利用，植物往往会因为缺乏铁营养而延迟固氮的起始。本研究中5株固氮菌均具有产铁载体的能力，产铁载体能力最强的是NY-6号菌株。Xing等[35]对甘蔗茎中固氮细菌的促生能力研究中表明碳、氮源的供应会影响细菌的生长速度和固氮酶活性，本研究通过对5株固氮细菌的培养条件进行优化发现，5株固氮细菌的最适碳源分别是可溶性淀粉和蔗糖，最适氮源分别是牛肉膏和蛋白胨，这与任书娴等[36]对硬叶兜兰内生真菌YD-16号菌株的最适碳源及氮源的筛选结果存在一致性，但本研究结果表明5株固氮细菌对铵态氮（NH_4^+-N）和硝态氮（NO_3^--N）的利用率不高，而YD-16号菌株对NH_4^+-N和NO_3^--N则较高，这可能是细菌和真菌的氮源偏好不同所导致的，细菌可能对有机氮的利用效率更高，5株固氮细菌在分别以胰蛋白胨或尿素为唯一氮源时的生长量多数显著高于以$(NH_4)_2SO_4$和KNO_3分别为唯一氮源时的生长量，也证实了细菌可能对有机氮的利用率更高。

综上所述，岩生型硬叶兜兰内生固氮细菌种类多样，可能在硬叶兜兰氮吸收方面发挥了关键作用，本次所测的5株固氮细菌均具有分泌IAA、产铁载体的能力，其中NY-6、NG-6和NG-44号菌株的促生能力较为突出，可做进一步研究；从培养条件来看，5株固氮细菌对有机氮的利用率更高，表明这些细菌具有降解有机氮的功效，由此可推测硬叶兜兰吸收的一部分氮营养来源于有机质降解，下一步将从能有效降解有机氮的细菌筛选及其促生效应开展相关试验。

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