Isolation and Screening of IAA-Producing Bacteria from Waxy Sorghum Leaves and Their Plant Growth-Promoting Effects Postprint

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Abstract

Glutinous sorghum is an important raw material for Baijiu brewing, and its growth process requires substantial amounts of chemical fertilizers. As an environmentally friendly fertilizer, microbial agents possess broad application prospects. To develop microbial agents that promote glutinous sorghum growth, this study employed glutinous sorghum leaves as experimental material to isolate and screen microbial strains capable of producing the plant growth hormone IAA (indole-3-acetic acid). Phylogenetic analysis based on the conserved 16S rDNA sequences of the strains was conducted to determine their taxonomic status. The effects of the strains on glutinous sorghum seed germination were analyzed through seed soaking treatment with bacterial suspension, and their effects on sorghum seedling growth were evaluated via pot experiments. The results showed that: (1) Four IAA-producing microbial strains, designated HY1-1, HY1-2, HY1-3, and HY1-4, were isolated and screened from glutinous sorghum leaves. Among them, strain HY1-1 exhibited the highest IAA production per unit concentration at 2.56 mol · L-1. (2) Phylogenetic analysis of the strains' 16S rDNA using Bayesian inference tree revealed that all four strains belonged to Bacillus subtilis. (3) Strains HY1-1, HY1-2, HY1-3, and HY1-4 all promoted glutinous sorghum seed germination. Compared with the control group, the germination rate of glutinous sorghum seeds soaked with bacterial suspension significantly increased by 40.04%~165.52%, with HY1-1 demonstrating the most pronounced promotion effect, increasing seed germination rate by 165.52%. (4) Strain HY1-1 was selected for pot experiments. Thirty days after inoculating B. subtilis HY1-1 at the roots of glutinous sorghum seedlings, seedling height significantly increased by 29.17% and total phosphorus content significantly increased by 5.12%; available nitrogen in the rhizosphere substrate significantly increased by 31.70%, and available phosphorus significantly increased

by 28.88%. In summary, the glutinous sorghum leaf endophyte B. subtilis HY1-1 can promote glutinous sorghum plant growth through secreting plant growth hormone IAA and providing nutrient elements for plants. This study provides germplasm resources for further development of microbial agents promoting glutinous sorghum growth.

Full Text

Isolation and Screening of IAA-Producing Bacteria from Glutinous Sorghum Leaves and Their Plant Growth-Promoting Function

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Abstract

Glutinous sorghum is an important raw material for baijiu production, and its cultivation typically requires substantial chemical fertilizer inputs. As an environmentally friendly alternative, microbial inoculants offer promising prospects for sustainable agriculture. This study aimed to develop microbial agents that promote glutinous sorghum growth by isolating and screening endophytic strains capable of producing indole-3-acetic acid (IAA), a key plant growth hormone. Using glutinous sorghum leaves as experimental material, we isolated four IAA-producing strains designated HY1-1, HY1-2, HY1-3, and HY1-4. Phylogenetic analysis based on 16S rDNA conserved sequences identified all four strains as *Bacillus subtilis*. The effects of these strains on seed germination were evaluated through bacterial suspension soaking treatments, while their impact on seedling growth was assessed via pot experiments.

The results demonstrated: (1) Strain HY1-1 exhibited the highest IAA production capacity at 2.56 $mol \cdot L^{-1}$ per unit concentration. (2) All four strains significantly promoted glutinous sorghum seed germination, increasing germination rates by 40.04% to 165.52% compared to the control group, with HY1-1 showing the most pronounced effect (165.52% increase). (3) In pot experiments, inoculation with *B. subtilis* HY1-1 for 30 days significantly increased seedling height by 29.17% and total phosphorus content by 5.12%. Additionally, available nitrogen in the rhizosphere substrate increased by 31.70%, and available phosphorus increased by 28.88%. These findings indicate that the leaf endophyte *B. subtilis* HY1-1 promotes glutinous sorghum growth through IAA secretion and enhanced nutrient provision. This study provides valuable germplasm resources for developing microbial inoculants specifically tailored for glutinous

sorghum cultivation.

Keywords: plant endophytic bacteria; isolation and screening; plant hormone IAA production; seed germination; glutinous sorghum growth promotion

Introduction

Glutinous sorghum, characterized by its high amylopectin content and strong viscosity after cooking, serves as a crucial raw material for high-quality baijiu production (Ding Yanqing et al., 2019). While fertilizer application is essential for achieving high sorghum yields—with nitrogen being the primary yield-determining factor followed by phosphorus—continuous heavy fertilizer inputs lead to reduced nutrient use efficiency, soil degradation, and water pollution (Chen Tongbin et al., 2002; Dong Erwei et al., 2012). Microbial organic fertilizers offer a sustainable alternative by increasing nutrient supply, promoting plant growth, enhancing yield and quality, improving the agricultural ecological environment, reducing plant diseases and pests, and decreasing chemical fertilizer requirements, thereby playing an increasingly important role in sustainable agricultural development (Li Xiqiang et al., 2013).

Plant endophytes reside within roots, stems, leaves, bark, flowers, and seeds, actively promoting host plant health and development (Dudeja et al., 2011; Santoyo et al., 2016). Leaf endophytes are acquired primarily through vertical and horizontal transmission (Balint et al., 2013), with leaf tissues providing a rich microenvironment that harbors abundant microbial resources (Van denkoornhuyse et al., 2015). In this mutualistic relationship, host plants provide continuous nutrient supply to endophytes, which in return enhance plant growth directly or indirectly through phosphate solubilization, nitrogen fixation, IAA production, siderophore synthesis, and ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity (Cueva-Yesquén et al., 2021). Through long-term coevolution, endophytic bacteria have developed intimate relationships with their host plants, making them superior competitors against other microbial formulations. Their rational application can reduce environmental pollution from chemical pesticides and fertilizers while maintaining ecological balance (Liang Zhichao et al., 2019).

IAA, an indole derivative in the auxin family, serves as a primary hormone regulating various developmental and physiological processes in plants (Nutaratat et al., 2015). It stimulates cell division, elongation, and differentiation, modulates phototropic and gravitropic responses, and regulates leaf abscission and fruit maturation (Teale et al., 2006). Consequently, IAA production capacity is considered a key indicator of plant growth-promoting potential in endophytic bacteria. This study utilized the Hongyingzi glutinous sorghum variety—widely used in sauce-flavor baijiu production—to isolate leaf endophytes using LB medium. Strain IAA production was detected using Salkowski reagent, molecular identification was performed through 16S rDNA sequencing, and plant

growth-promoting efficacy was evaluated through comprehensive seed germination and pot experiments. The objective was to obtain microbial strains that effectively promote glutinous sorghum growth and to preliminarily explore their growth-promoting mechanisms.

Materials and Methods

1.1 Experimental Materials

The experimental glutinous sorghum (*Sorghum bicolor* var. *glutinosa*) was the Hongyingzi variety used for sauce-flavor baijiu production in Maotai Town, Guizhou Province, obtained from the Renhuai Fengyuan Organic Sorghum Breeding Center.

1.2 Isolation and Purification of Leaf Endophytes

Ten grams of glutinous sorghum seedling leaves were cut into small pieces with sterile scissors and surface-sterilized by sequential immersion in 70% ethanol for 2 minutes, 5% sodium hypochlorite for 5 minutes, and 70% ethanol again for 2 minutes, followed by three rinses with sterile water. The sterilized leaves were further cut into fragments and transferred to 90 mL sterile water, then vortexed for 30 minutes to create a master bacterial suspension. This suspension was serially diluted to 10^{-1}, 10^{-2}, and 10^{-3} concentrations. One hundred microliters from each dilution were spread onto LB solid plates and incubated inverted at 30°C for 72 hours. Single colonies were selected and purified using the quadrant streak method, with pure cultures preserved in 30% glycerol at -80°C.

1.3 Determination of IAA Production Capacity

Strains were inoculated into R2A liquid medium and incubated at 30°C with shaking at 150 rpm for 4 days. The optical density of the bacterial suspension was measured at λ=600 nm. The culture was then centrifuged at 5,000 rpm for 10 minutes. Ten milliliters of supernatant were mixed with an equal volume of Salkowski colorimetric solution and incubated in darkness for 30 minutes before measuring absorbance at λ=530 nm. IAA concentration was calculated using a standard curve, and production capacity was expressed as IAA produced per unit bacterial concentration (OD_{600} = 1) (He Jianqing et al., 2019).

1.4 Molecular Biological Identification

Genomic DNA was extracted using a modified sodium lauryl sulfate method. The 16S rDNA fragment was amplified using universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACT-3'). PCR products were sequenced by a biotechnology company (Shanghai) and compared against the NCBI GenBank database. High-homology reference sequences were downloaded, aligned using

ClustalW 1.6 in MEGA11 software, and the optimal model for Bayesian inference was calculated using ModelTest3.7 combined with paup*4.0v10b based on the Akaike Information Criterion (Posada & Crandall, 1998; Cummings, 2004). A Bayesian inference tree was constructed using MrBayes 3.1.2 with the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm, employing one cold chain and three heated chains. Two independent runs were performed for 2,000,000 generations, sampling every 1,000 generations. After convergence (average standard deviation of split frequencies < 0.01), 2,000 trees were obtained. The first 25% (500 trees) were discarded as burn-in, and the remaining 75% (1,500 trees) were used to generate the 50% majority-rule consensus tree as the final 16S rDNA phylogenetic tree (Ronquist & Huelsenbeck, 2003; Huelsenbeck & Ronquist, 2011).

1.5 Seed Germination Experiment

1.5.1 Bacterial Suspension Preparation Single colonies were selected from LB plates and inoculated into LB liquid medium, incubated at 30°C with shaking at 150 rpm for 3 days. Cells were harvested by centrifugation at 5,000 rpm for 10 minutes, washed three times with sterile water, and resuspended to a final optical density of $OD_{600} = 0.5$–0.6.

1.5.2 Seed Surface Sterilization Uniform, plump glutinous sorghum seeds were surface-sterilized by sequential treatment with 70% ethanol for 2 minutes, 5% sodium hypochlorite for 5 minutes, and 70% ethanol for another 2 minutes, followed by 3–5 rinses with sterile water.

1.5.3 Bacterial Suspension Soaking One hundred milliliters of bacterial suspension were used to soak sorghum seeds for 6 hours at room temperature in darkness (wrapped in aluminum foil). After soaking, the suspension was discarded, and seeds were rinsed 3–5 times with sterile water before being placed on water agar plates (1.4% agar) using sterile forceps. Plates were incubated inverted at 28°C. The control group was treated with an equal volume of distilled water. Each treatment used 100 seeds with three replicates.

1.5.4 Data Collection and Analysis Germination counts were performed on days 3 and 7 to calculate germination potential and germination rate (Zheng Qingzhong et al., 2016) using the following formulas:

$$\text{Germination potential} = \frac{\text{Number of germinated seeds at initial stage (day 3)}}{\text{Total number of tested seeds}} \times 100\%$$

$$\text{Germination rate} = \frac{\text{Number of germinated seeds at final count (day 7)}}{\text{Total number of tested seeds}} \times 100\%$$

1.6 Pot Experiment

1.6.1 Seed Pre-germination Surface-sterilized sorghum seeds (as described in 1.5.2) were placed on water agar plates and incubated inverted at 28°C for 48 hours to promote germination.

1.6.2 Experimental Setup Perlite and vermiculite were mixed at a 1:2 ratio and placed in plastic pots (17.5 cm height × 11.5 cm base diameter × 16.5 cm top diameter). Pots were sealed with newspaper and sterilized at 121°C for 30 minutes. Germinated seeds were planted at 20 seeds per pot, with 10 pots per treatment.

1.6.3 Inoculation Procedure Bacterial suspensions were prepared as described in 1.5.1. When sorghum seedlings emerged, each pot received 10 mL of bacterial suspension, while control pots received an equal volume of sterile water. Modified Hoagland nutrient solution was added as needed (Haibo Biological, Hoagland nutrient solution without calcium nitrate, product number HB8870-1, supplemented with NH_4NO_3 0.92 g/L and $CaCl_2$ 0.64 g/L).

1.6.4 Sampling and Parameter Measurement After 30 days of inoculation, sorghum seedlings were carefully removed from the substrate, and roots were washed with running water. Surface moisture was absorbed with filter paper before measuring plant height and fresh weight. Whole plants were digested with sulfuric acid-hydrogen peroxide for nutrient analysis. Total nitrogen content was determined by the Kjeldahl method (Ma Zongqi et al., 2014), and total phosphorus content was measured by the molybdenum-antimony colorimetric method. Soil available nitrogen was determined by the alkaline hydrolysis diffusion method (Yang Qinghua, 2018), and available phosphorus was extracted with sodium bicarbonate solution and measured by the molybdenum-antimony colorimetric method.

1.7 Statistical Analysis

All data were analyzed and graphed using Origin 2019 software. Statistical significance was determined at $P < 0.05$.

Results

2.1 Strain Isolation and Identification

Four bacterial strains were isolated from glutinous sorghum leaves and designated HY1-1, HY1-2, HY1-3, and HY1-4. [Figure 1: see original paper] illustrates their colony morphologies on LB plates. HY1-1 formed circular, yellow, opaque colonies with smooth surfaces that were easily picked. HY1-2 produced larger, slightly reddish colonies with smooth, opaque surfaces. HY1-3 exhibited

light yellow, smooth, opaque colonies, while HY1-4 displayed white, needle-shaped colonies with smooth, opaque surfaces.

Phylogenetic analysis based on 16S rDNA sequences was performed to determine taxonomic status. As shown in [Figure 2: see original paper], the Bayesian inference tree clustered HY1-1, HY1-2, HY1-3, and HY1-4 with *Bacillus subtilis* strain DSM 10 with 98% confidence, confirming that all four strains belong to *Bacillus subtilis*. Consequently, they were named *B. subtilis* HY1-1, HY1-2, HY1-3, and HY1-4.

2.2 IAA Production Capacity

An IAA standard curve was prepared using 17.50 mg of analytical-grade IAA dissolved in minimal ethanol and diluted to 100 mL to obtain a 1 mmol $\cdot$ L^{-1} standard solution. Absorbance values were plotted against IAA concentrations to establish the standard curve, which was used to calculate IAA concentrations in culture supernatants. presents the IAA production capacities normalized to $OD_{600} = 1$. HY1-1 exhibited the highest production at 2.56 mol $\cdot$ L^{-1}, followed by HY1-3 at 2.29 mol $\cdot$ L^{-1}, while HY1-2 showed the lowest production at 1.43 mol $\cdot$ L^{-1}.

2.3 Effects on Seed Germination

As shown in [Figure 3: see original paper]A, the control group exhibited a germination potential of 17.67%. Treatment with *B. subtilis* HY1-1 suspension significantly increased germination potential to 37.33% (111.26% increase). HY1-2 treatment showed no significant change (18.33%), while HY1-3 modestly increased germination potential to 21.33% (7.64% increase). Interestingly, HY1-4 treatment slightly decreased germination potential to 16% (5.88% decrease). [Figure 3: see original paper]B demonstrates that the control germination rate was 18.33%. All bacterial treatments significantly enhanced germination rates: HY1-1 increased it to 48.67% (165.52% increase), HY1-2 to 37.67% (105.51% increase), HY1-3 to 41.33% (125.48% increase), and HY1-4 to 25.67% (40.04% increase). These results confirm that all four *B. subtilis* strains positively promoted glutinous sorghum seed germination, with HY1-1 showing the most significant effect.

2.4 Effects on Seedling Growth

Based on the superior performance in previous experiments, HY1-1 was selected for pot experiments. [Figure 4: see original paper]A shows that after 30 days of inoculation, HY1-1-treated seedlings exhibited more vigorous growth with robust stems compared to the uninoculated control. Random selection of 30 seedlings from each group revealed that control plants averaged 15.53 cm in height, while treated plants reached 20.06 cm, representing a significant 29.17% increase ($P < 0.05$). These results demonstrate that *B. subtilis* HY1-1 effectively promotes glutinous sorghum seedling growth.

2.5 Effects on Plant Nutrient Content and Rhizosphere Substrate

2.5.1 Total Plant Nitrogen and Substrate Available Nitrogen As shown in [Figure 5: see original paper]A, total nitrogen content in control plants was 24.50 $g \cdot kg^{-1}$, while HY1-1-inoculated plants contained 24.60 $g \cdot kg^{-1}$, with no significant difference between groups. However, [Figure 5: see original paper]B reveals that available nitrogen in the rhizosphere substrate increased significantly from 20.80 $mg \cdot kg^{-1}$ in controls to 27.40 $mg \cdot kg^{-1}$ in the treatment group (31.70% increase, $P < 0.05$). These results indicate that HY1-1 inoculation enhanced available nitrogen in the rhizosphere without significantly affecting nitrogen uptake by the plants.

2.5.2 Total Plant Phosphorus and Substrate Available Phosphorus [Figure 6: see original paper]A shows that total phosphorus content in control plants was 3.89 $g \cdot kg^{-1}$, which increased significantly to 4.10 $g \cdot kg^{-1}$ in HY1-1-treated plants (5.12% increase, $P < 0.05$). Similarly, available phosphorus in the rhizosphere substrate increased from 10.77 $mg \cdot kg^{-1}$ in controls to 13.88 $mg \cdot kg^{-1}$ in the treatment group (28.88% increase, $P < 0.05$). These findings demonstrate that HY1-1 inoculation not only increased substrate phosphorus availability but also enhanced phosphorus uptake by sorghum plants.

Discussion and Conclusion

The Bacillaceae family comprises aerobic, spore-forming Gram-positive rods with diverse physiological characteristics, wide distribution, and easy cultivation, making them the most extensively studied and applied group of plant endophytic bacteria (Gong Guoli et al., 2020). This study successfully isolated four endophytic strains from sorghum leaves, all identified as *Bacillus subtilis* with IAA production capacities ranging from 1.43 to 2.56 $mol \cdot L^{-1}$. These strains significantly improved glutinous sorghum seed germination rates, with HY1-1 demonstrating the most pronounced effects by substantially increasing seedling height. Similar findings have been reported for *B. subtilis* EA-CB0575, which increased potato dry weight by 34.60% through mechanisms involving IAA, siderophore, and nitrogenase synthesis—genes that exhibit conservation across *B. subtilis* genomes (Nicolás et al., 2020). This suggests that *B. subtilis* promotes plant growth through phosphate solubilization, nitrogen fixation, and siderophore production.

Previous studies have demonstrated that microbial inoculants containing *Bacillus* species significantly promote plant growth. For instance, Li Le et al. (2016) showed that rhizobial and composite microbial agents enhanced mung bean growth, while Alou et al. (2015) and Kotb et al. (2015) confirmed the plant growth-promoting and pathogen-inhibiting capabilities of *Bacillus* species. Wu Yuhong et al. (2023) reported that marine *Bacillus velezensis* promoted cucumber seedling growth through nitrogen fixation, ACC deaminase production, and

IAA synthesis. Zhang Rongsheng et al. (2018) found that *Bacillus amylolyticus* produced high levels of auxins, gibberellins, abscisic acid, and spermidine, promoting rice growth while providing biocontrol benefits. Liu Lufeng et al. (2020) isolated endophytic *B. subtilis* from sugarcane that enhanced physiological indicators in both maize and sugarcane. Our study extends these findings to glutinous sorghum, demonstrating the broad host range of *B. subtilis* and its potential application value in cultivating raw materials for baijiu production, thereby contributing to sustainable development in the liquor industry.

Although this study did not directly measure phosphate solubilization and nitrogen fixation capacities, the pot experiment results strongly suggest these functionalities. The significant increases in rhizosphere available nitrogen (31.70%) and available phosphorus (28.88%) following HY1-1 inoculation indicate that the strain possesses both nitrogen-fixing and phosphate-solubilizing abilities, thereby enhancing host plant nutrient acquisition. Nitrogen and phosphorus are critical for sorghum production, influencing not only yield but also grain starch composition (Cao Changlin et al., 2011). Low phosphorus stress negatively impacts sorghum dry weight, shoot biomass, chlorophyll content, and soluble protein levels (Ma Jianhua et al., 2013). Our results demonstrate that leaf-derived *B. subtilis* can effectively provide nitrogen and phosphorus nutrition to host plants, ensuring normal growth and development while offering an efficient screening approach for future strain development.

Future research should combine high-throughput sequencing with pure culture isolation to reveal the diversity and community composition of glutinous sorghum endophytes, enabling the selection of superior strains with multiple growth-promoting traits for specialized inoculant development. Building upon our preliminary exploration of strain HY1-1, subsequent studies should investigate the molecular mechanisms underlying sorghum's response to *B. subtilis* HY1-1 colonization to elucidate the deeper mechanisms of growth promotion, providing both technical insights and microbial resources for improving glutinous sorghum yield and quality.

This study yields three major conclusions: (1) Leaves represent an important source for isolating growth-promoting endophytes from glutinous sorghum; (2) The four isolated *B. subtilis* strains possess IAA production capacity (1.43–2.56 $\mathrm{mol \cdot L^{-1}}$) and significantly promote sorghum seed germination by increasing both germination potential and germination rate; (3) Among the tested strains, HY1-1 exhibits the strongest growth-promoting activity, significantly increasing plant height by 29.17%, rhizosphere available nitrogen by 31.70%, available phosphorus by 28.88%, and total plant phosphorus by 5.12%, providing valuable microbial resources for large-scale development of growth-promoting inoculants.

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Note: Figure translations are in progress. See original paper for figures.