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ORIGINAL ARTICLE

Endophytic bacteria from *Pinellia ternata*, a new source of purine alkaloids and bacterial manure

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Abstract

Context: *Pinellia ternata* (Thunb.) Berit., a perennial herb belonging to Araceae, is one of the few medicinal plants to produce purine alkaloids. It is speculated that endophytic bacteria from *P. ternata* may produce guanosine or inosine. However, there is no report about endophytic bacteria in *P. ternata*.

Objective: In this study, endophytic bacteria were isolated from *P. ternata* and examined for the first time. This study finds a novel way to increase the yield of *P. ternata* herb, and to provide some new alkaloid producers.

Materials and methods: Plant material includes leaves, tubers, and roots of cultivated and wild *P. ternata*. The dilutions were smeared onto beef extract-peptone medium and cultured at 28 °C in darkness for 48–72 h. Co-culture treatments were prepared by inoculating 100 mL liquid 1/2 MS medium with bacterial culture broth at concentrations of 0 (control), 0.5%, and 1.5% (v/v).

Results: Of the 34 endophytic bacterial colonies isolated from *P. ternata* leaves, roots, and tubers, five strains were able to produce purine alkaloids. Results from 16s rDNA sequence analysis indicated that the bacteria belonged to *Bacillus cereus*, *Aranicola proteolyticus*, *Serratia liquefaciens*, *Bacillus thuringiensis*, and *Bacillus licheniformis*. Co-culture with living *Serratia liquefaciens* cells increased PLB growth by 58–71%. Co-culture with living *Bacillus licheniformis* cells increased PLB growth by 4–11%.

Conclusion: This study provides a novel way for improving the yield of *P. ternata* herb, and for the production of purine alkaloids by the fermentation industry.

Keywords

Alkaloids, endophytic bacteria, guanosine, herb, inosine, *Pinellia ternata*, secondary metabolites

History

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Introduction

Pinellia ternata (Thunb.) Berit., a perennial herb belonging to Araceae, has been used in Chinese traditional medicine for over 2000 years. The herb is used for its anti-emetic, analgesic, and sedative effects (Han et al., 2006). Recent reports indicate that *P. ternata* has anti-anxiety, anticancer, and anti-inflammation effects as well as the ability to induce abortion in early pregnancy (He et al., 2007; Hu et al., 2008). Alkaloids are the main biologically active compounds in *P. ternata*. Two purine alkaloids, guanosine and inosine, have been isolated from *P. ternata*. Guanosine is a water-soluble compound that is commonly found in medicinal herbs (Zhang et al., 2005). In contrast, inosine is generally found in animals or microorganisms (Wu et al., 2003). Both alkaolids are known to have antitumor and antivirus activities (Kinahan et al., 1981).

Plant endophytes can produce the same or similar secondary metabolites as their host plants (Li et al., 1998). It is speculated that endophytic bacteria from *P. ternata* may produce guanosine or inosine. However, there is no report about endophytic bacteria in *P. ternata*.

Demand for *P. ternata* is increasing in recent years. The supply cannot keep pace with the increasing demand. It was reported plant endophytes could promote the growth of their host plant (Hinton & Bacon, 1995). We speculated endophytes of *P. ternata* may enhance the growth of the herb.

In this study, endophytic bacteria were isolated from *P. ternata* for the first time. Then the bacteria were screened to determine their capacity for producing guanosine or inosine. Strains which produced either of the two alkaloids were identified through 16s rDNA sequencing. Two bacteria strains, *Serratia liquefaciens* and *Bacillus licheniformis*, were co-cultured with PLBs of *P. ternata* to test their effect on the growth of PLBs. This study attempts to find a novel way to increase the yield of *P. ternata* herb, and to provide some new alkaloid producers.

Materials and methods

Plant material

Plant material includes both cultivated and wild *P. ternata*. Cultivated tubers, 0.5–1.5 cm in diameter, were collected in

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August and September 2010, from *P. ternata* grown in experimental fields at Northwest A&F University, Yangling, China. Wild tubers, 1.0–1.5 cm in diameter, were collected in October 2010, from *P. ternata* grown in Zhenping County, Ankang, China. *Pinellia ternata* is identified by Professor Yuejin Zhang. A voucher specimen of the plant is deposited at the Department of Life Science, Northwest A & F University.

Isolation of endophytic bacteria from *P. ternata*

Fresh leaves, tubers, and roots were rinsed with tap water and air-dried. Roots, leaves, and tubers were cut into 1 cm, $1 \times 1\,cm^2$, and $0.5 \times 1 \times 1\,cm^3$ segments, respectively. The tissue segments were surface-sterilized in 75% (v/v) ethanol for 1 min, 0.1% (m/v) mercuric chloride solution for 4–6 min (roots and leaves 4 min, tubers 6 min), and rinsed three times with sterile distilled water, then drained. The tissues (3 g for leaves or roots, 7 g for tubers) were ground in 10 mL distilled water and then 10-fold serial dilutions (10^{-1}–10^{-4}) of the homogenates were prepared using distilled water. The dilutions were smeared onto beef extract-peptone medium and cultured at 28 °C in darkness for 48–72 h. Morphologically, distinct colonies on the culture medium were selected, purified, and then conserved at 4 °C. The control in the experiment was the water which had been used to rinse the sterilized plant material for the last time. An aliquot of this water was smeared onto beef extract-peptone medium and cultured at 28 °C. No microbes were observed growing on the culture medium, indicating that the plant materials had been completely surface-sterilized. Colonies that grew from the plant homogenates must have been endophytic bacteria (Chen et al., 2006; He et al., 2002).

Selection bacteria production alkaloids of *P. ternata*

After selection and purification, the bacteria were grown in shaker flasks containing 100 mL liquid Luria–Bertani (LB) medium. The flasks were shaken on an orbital shaker (250 rpm) in darkness at 28 °C for 3 d. At the end of the incubation, Fermentation broths were concentrated, bacterial cells were broken by ultrasonication, and then the fermentation broths were homogenized and centrifuged at 10 000 rpm for 10 min. Chitosan (0.03% v/v) was added to the supernatant to precipitate carbohydrates and then the liquids were put in a refrigerator at 4 °C for 12 h. The supernatants were filtered through a 0.45 μm microfiltration membrane and the subsequent filtrates were analyzed by HPLC. The control in the experiment was the flasks containing 100 mL liquid Luria–Bertani (LB) medium without bacteria.

Guanosine and inosine contents of the fermentation broths were determined using a standard HPLC system equipped with a 1525 binary pump and a 2996 photodiode array detector. The column was a Sunfire C-18 reverse-phase column (Waters Corporation, Milford, MA), 250 mm × 4.6 mm i.d., 5 μm particle diameter, 100 Å average pore size with a mobile phase composed of methanol/water (2:98, v/v). The flow rate was 1.0 mL min^{-1} and the column temperature was 30 °C. Alkaloid content was quantified by UV absorption at 248 nm for inosine and 254 nm for guanosine. Alkaloid contents were calculated according to the standard curves of reference standards.

Identification of endophytic bacteria

Endophytic bacteria were identified by 16s rDNA sequence analysis. The work was done by Shanghai Shengong Company, which is one of the most authoritative bacteria identification institute in Shanghai, China. After identification, the bacterial strains were deposited at the Microbe Research Center, Northwest A & F University, Yangling, China.

Effect of endophytic bacteria on PLB growth

For the co-culture treatment, culture broth containing living cells was prepared by growing *Serratia liquefaciens* and *Bacillus licheniformis* cells in shaker flasks containing liquid LB medium (pH 7.0). The flasks were shaken on an orbital shaker (250 rpm) in darkness at 30 °C for 24 h. The optical density (OD) in the medium reached a maximum value of 0.8 (pH 6.5).

Co-culture treatments were prepared by inoculating 100 mL liquid 1/2 MS medium with bacterial culture broth at concentrations of 0 (control), 0.5, and 1.5% (v/v). After the culture medium was prepared, we transferred 3.0 g of 5-week-old undifferentiated PLBs to each flask. The flasks were placed on an orbital shaker (110 rpm) for 35 d at 25 °C and 12 h white fluorescent light (1200 l×) per day.

The growth index (GI) of the PLBs was calculated over a 35-d culture period by dividing the final PLB fresh weight by the initial PLB fresh weight. The differentiation rate (DR) of the PLBs was calculated by dividing the number of differentiated PLBs by the total number of PLBs (differentiated plus undifferentiated PLBs).

Statistical analysis

All data presented are the average of three replicates. Statistical analysis was confirmed by Duncan's multiple range test (DMRT) using SAS 8.0 software for windows (SAS Inc., Cary, NC).

Results and discussion

Homogenates from surface sterilized cultivated and wild *P. ternata* leaves, tubers, and roots were cultured on the beef extract-peptone medium. We identified 34 morphologically distinct colonies growing on the medium. Among these, 14 colonies were isolated from tubers, nine colonies were isolated from roots, and 11 colonies were isolated from leaves. The colonies were cultured separately in the liquid LB medium for 3 d and then water extracts from the fermentation broth were analyzed by HPLC. Results showed that bacteria from five colonies produced guanosine and inosine (Figure 1). The 16s rDNA sequence analysis indicated that the bacteria belonged to *Bacillus cereus*, *Aranicola proteolyticus*, *Serratia liquefaciens*, *Bacillus thuringiensis*, and *Bacillus licheniformis.*

It is very important to isolate endophytes correctly. Several sterilants were tested for *P. ternata*. It showed surface sterilization was thoroughly by 75% (v/v) ethanol and 0.1% (m/v) mercuric chloride solution. Sterilizing periods were tested. Some endophytes may be killed with a time longer than 7 min, while surface microbes cannot be killed

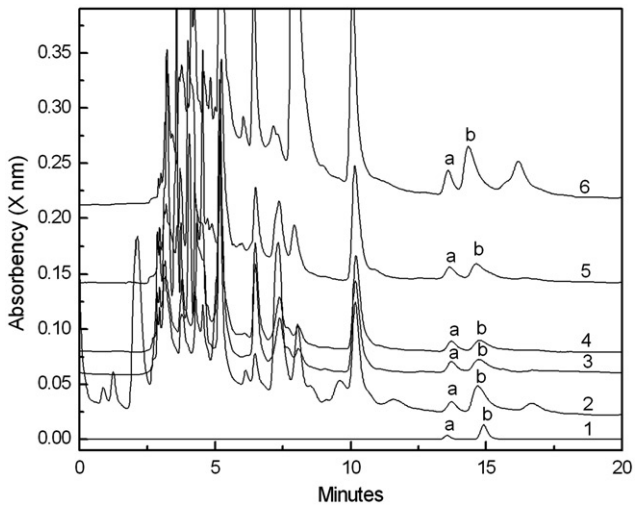

Figure 1. Guanosine and inosine content of fermentation broths containing endophytic bacteria isolated from *P. ternata*. Alkaloid content was quantified by UV absorption at 248 nm for inosine and 254 nm for guanosine, but only absorption at 254 nm is shown. (1) Standard solution; (2) *Bacillus thuringiensis*; (3) *Aranicola proteolyticus*; (4) *Bacillus cereus*; (5) *Serratia liquefaciens*; and (6) *Bacillus licheniformis*. Peak a: inosine; Peak b: guanosine.

Table 1. Alkaloid production by endophytic bacteria isolated from *P. ternata*[a].

Fermentation broth	Guanosine/(mg/L)	Inosine/(mg/L)
Control	0.0	0.0
Bacillus cereus	2.360 ± 0.051	2.701 ± 0.064
Aranicola proteolyticus	3.652 ± 0.047	3.058 ± 0.033
Serratia liquefaciens	5.143 ± 0.073	3.886 ± 0.040
Bacillus thuringiensis	8.082 ± 0.046	2.858 ± 0.052
Bacillus licheniformis	8.718 ± 0.059	3.709 ± 0.044

[a]Data are expressed as means ± standard deviations from three independent experiments, each with three replicates.

thoroughly with a time shorter than 4 min. It took a longer sterilizing time for tubers than for leaves and stems. It showed a sterilizing period within 4–6 min was suitable.

A comparison of guanosine and inosine production by the endophytic bacteria is shown in Table 1. All the five bacterial strains produced larger amounts of guanosine when compared with inosine production. There was a relatively wide range in the amount of guanosine produced by the bacteria. Guanosine production was greatest for strains belonging to *Bacillus thuringiensis* and *Bacillus licheniformis.* In contrast, inosine production was nearly the same among the five strains.

It should be noted that alkaloid production by these five endophytic strains was low, compared with that of 1 g/L inosine by mutant of *E. coli* (Matsui et al., 2001), 6 g/L inosine by *B. subtilis* (Asahara et al., 2010), and 20 g/L guanosine by mutants of *B. subtilis* (Kuninaka, 2008). The production of guanosine and inosine by *Bacillus subtilis* has been increased by optimizing fermentation conditions, breeding new bacterial strains, and regulating the metabolic pathway of the bacteria (Bai et al., 2003; Liu et al., 2004; Qian et al., 2003; Zhu et al., 2004). Similar methods might be employed to increase guanosine or inosine production from the bacterial strains isolated in our study.

Table 2. Effect of *Serratia liquefaciens* and *Bacillus licheniformis* on PLB proliferation and differentiation.

	Serratia liquefaciens		*Bacillus licheniformis*	
Treatments	GI[a]	DR[b]	GI	DR
Live bacteria control	3.11	50	3.23	50
0.5%	4.91	98	3.35	29
1.5%	5.31	93	3.57	13

[a]GI (growth index) = final PLB fresh weight divided by initial PLB fresh weight.
[b]DR (differentiation rate) = number of differentiated PLBs divided by the total number of PLBs.

The effects of co-culture with living cells on PLB growth and differentiation are shown in Table 2. Co-culture with living *Serratia liquefaciens* cells increased PLB growth by 58–71% and PLB differentiation by 81–96%. Co-culture with living *Bacillus licheniformis* cells increased PLB growth by 4–11% and decreased PLB differentiation by 42–74%.

PLBs of *P. ternata* are a kind of highly differentiated tissue. PLBs are similar to field-grown tubers morphologically and in microstructure (Liu et al., 2010). Both the two bacterial strains, *Serratia liquefaciens* and *Bacillus licheniformis*, increased the growth rate of PLBs. It indicates that endophytic bacteria may promote the growth of *P. ternata* field-grown tubers. Further studies may pay attention to bacterial manure to improve the yield of *P. ternata* herb.

Conclusions

In summary, we isolated endophytic bacteria of *P. ternata* for the first time. Among the 34 bacterial colonies isolated, five endophytic bacteria strains had the ability to produce the same alkaolids as their host plant. These bacteria were identified by 16s rDNA sequence analysis. Further studies may focus on improving guanosine and inosine yields in fermentation broth. Two bacterial strains, Serratia liquefaciens and Bacillus licheniformis, may be studied as manure to improve field-grown P. ternata yield.

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Declaration of interest

The authors report that they have no conflicts of interest. This work was supported by a Scientific Research Program Funded by Shaanxi Provincial Education Department (Fund number appended later).

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