Root endophytes enhance stress-tolerance of *Cicuta virosa* L. growing in a mining pond of eastern Japan

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Short running title: Endophyte promote growth, Zn uptake and tolerance

Abstract

Cicuta virosa L. plants can grow in a heavy-metal subjected pond at the Hitachi mine, eastern Japan. They accumulate heavy-metal elements especially with high concentration of zinc (Zn) in their roots. We focused that root bacterial endophytes should play a role in the heavy-metal uptake of plants and provide a heavy-metal tolerance with plants. Our purpose in the present study was to clarify the effects of endophytes 1) on Zn accumulation in *C. virosa* roots, 2) on growth of *C. virosa* seedlings, and 3) on heavy-metal tolerance of *C. virosa* plants. Root endophytic *Pseudomonas putida* and *Rhodopseudomonas* sp., which induced the high production of Zn-chelating compounds, were selected for the seedling-inoculation test. The results of the inoculation test demonstrated that both strains of endophytes increased Zn accumulation in *C. virosa* roots by solubilizing Zn in the soil. Both strains also increased the growth of seedlings by possible production of indole-3-acetic acid in the plant. The heavy-metal tolerance of *C. virosa* seedlings were likely promoted by producing metal-chelating compounds that detoxify the metals in the plant tissues, and by decreasing in the heavy-metal contents in the tissues *via* the rapid seedling growth. Thus, such mutualistic interactions between plants and bacteria contribute to the persistence of *C. virosa* in the severe environment.

Keywords: Cicuta virosa L., heavy metal, mine, pond, endophytes.

Introduction

There are various potential benefits for the host plant in mutualistic interactions with symbiotic microbes. Especially, endophytes can suppress the diseases, and induce the tolerance to environmental stress and then enhance plant growth (Ma *et al.*, 2011). Such biological interactions associate with plant-microbe coevolution and the material cycle in the ecosystems (Smith & Read 2008). Recently, it has been reported that root bacterial endophytes could play important roles in the heavy-metals uptakes of plants from the substrates and then stress-tolerance of plants (reviewed in Zaets & Kozyrovska 2012). Among several mechanisms in such roles, the metal-chelating compounds produced by microbes attract the attentions with respect to a heavy-metal tolerance in plants from the field of chemical ecology.

Often, specific types of plant community are observed on the veins of heavy-metal elements, which often contain high concentration of heavy metals in the substrates. The plant species composing such community should have been adapted to the heavy-metal environments (see Larcher 2003). The mechanisms of heavy-metal tolerance in plants can be classified into mainly following two types: 1) exclusions of the metals from their cells and 2) detoxifications of the metals by producing detoxicants *via* the formations of metal-complexes in the vacuoles (Hall 2002). For example, using several plant species called as the hyperaccumulators (e.g. *Thlaspi caerulescens*, *Brassica naps*, *Brassica juncea*), it has been documented that they showed a heavy-metal tolerance due to the detoxification mechanisms even if they accumulate high concentration of heavy metals in their tissues (Brooks *et al*. 1998; McGrath & Zhao 2003). The plants growing on veins and old mines should have such mechanisms to survive in the metallic habitats. In the present study, we focused the roles of endophytes in their heavy-metal tolerance.

Some types of rhizospheric bacteria increase heavy-metal uptake into plant cells by producing metal-chelating compounds like a siderophore, because most of heavy metals are immobilized in soil and these metal-chelating compounds can solubilize the metals from the soil. If the metal-chelating compounds are produced into the root tissues by the endophytes, these compounds would be helpful to detoxify heavy-metals in the plant cells. Therefore, endophytes that produce metal-chelating compounds could enhance the uptake of heavy metals into the plant cells as well as the heavy-metal tolerance of plants. In supporting this view, on the enhancements of heavy-metal accumulation in the plants, Whiting *et al.* (2001) reported that Zn-chelating compounds producing endophytes (*Microbacterium saperdae*, *Pseudomonas monteilii*, and *Enterobacter cancerogenus*) increased Zn uptake in a hyperaccumulator *Thlaspi caerulescens* aboveground parts and roots. That is, the plant ability to accumulate heavy metals in their tissues is enhanced by inoculation with endophytes that solubilize heavy metals in the soil. Thus, we regarded the plant-microbe interaction in the rhizosphere as significant roles when the plants survive in the heavy-metal subjected habitats.

The Japanese Islands have occasionally a wealth of vein deposits because a lots of mines were operated to refine ores into metals in past times (1905 -1976). At Hitachi mine in eastern Japan (Ibaraki Prefecture, the Honshu Island, Japan), the soil contains high concentrations of heavy metals, especially, Zn, Cu, Pb, and Cd (Kubota *et al.* 1986; Kamiga and Tejiri 2003) and it is still difficult for plants to grow there. However, a perennial helophyte *Cicuta virosa* L. (*Apiaceae*) was found as the predominant species in a small pond at the mine. *Cicuta virosa* plants usually grow in the swampy wet habitats (e.g. ponds, moors and paddy fields) and has fascicled tuberous root system as a thick rhizome. The root systems contain poisonous materials (Schep *et al.* 2009) and the

poisonous polyacetylenes in this species have been well studied in the fields of pharmacology and medical sciences (Jacobson 1915; Appendino *et al.* 2009). On the other hand, the functions of their root systems, especially the mechanisms to survive the mining sites, have not been investigated even though *C. virosa* is an species that greatly reduce the number in several areas (e.g. Kumamoto and Tokyo) of Japan (Review committee on rare and wild flora and fauna in Kumamoto prefecture 2009; Japan wildlife research center 2010).

Our preliminary chemical analysis clarified that *C. virosa* accumulates high concentration of Zn especially in the roots (7,542 mg/kg), which is an enough to induce toxic symptoms in other plant species (Nagata 2014), and this Zn concentration is more than 20 times to reach the toxic level of crops. Several wetland plant species (including the *Apiaceae*, *Brassica*, and *Polygonaceae*) accumulate heavy metals primarily in roots at the mine sites, but the metal concentrations in the roots are not extreme high (23.5 -117.1 mg/kg), though their heavy-metal uptakes show seasonal variations (Weis and Weis 2004; Brunetti *et al.* 2009). However, the mechanisms of heavy-metal tolerance in these plants have not been investigated. We focused in the reason why *C. virosa* plants can survive heavy-metal subjected soil even with highly accumulated Zn in their roots. Considering mutualism between the plants and the endophytes, we set up following hypothesis; the endophytes to produce Zn-chelating compounds increase the Zn uptake into the roots and then enhance the heavy-metal tolerance of *C. virosa* plans *via* detoxifications of Zn in their cells. Our purpose of present study was to clarify the effects of endophytes 1) on Zn accumulation in *C. virosa* roots, 2) on growth of *C. virosa* plants, and 3) on heavy-metal tolerance of *C. virosa via* the inoculation test of endophytes to the seedlings. We isolated the bacterial endophytes from *C. virosa* roots.

Especially, two endophytes *Pseudomonas putida* and *Rhodopseudomonas* sp. with high production ability of Zn-chelating compounds in the Zn-solubilization test were selected for the inoculation test. In order to exclude the other microbial contamination, the axenic *C. virosa* seedlings developed in the sterilized system were used in this test.

Materials and methods

Study site, sample collection and soil properties

Our study site, a pond at Hitachi mine is located in the north of Ibaraki Prefecture, Japan (36°27′N, 140°38′E). On the mountainside, there are two different sizes of ponds, which are connected though small flow channel. Water flow generate from the bigger pond (approximately 33 x 66.4 m) to the other pond (small pond; 5 x 5 m), where *Cicuta virosa* L. (*Apiaceae*) plants grows as the predominant species *via* vegetative propagation. The small pond is surrounded by *Clethra barvinervis* Seib. et Zucc. and kept to be under tree-shaded condition. Population density of *C. virosa* over the small pond was 127 ± 16 individuals/m^2 (mean ± SD, N = 506) in April, 2007. Samples of *C. virosa* plants, pond soil (sandy loam), and water were collected from the pond once a month from July to September 2006 and April to May 2007 using random number list. The pH of pond soil was 5.45 ± 0.01 (soil :water ratio, 1:2.5). The following soil properties were determined using elemental analysis (Vario MAX CN, Elementar Analysensysteme GmbH, Hanau, Germany) : total C 120 g/kg, total N 7.0 g/kg, coarse sand 34.9%, fine sand 41.2%, silt 11.1%, clay 12.8%, and cation exchangeable capacity (CEC) 28.4 cmol (+)/kg.

Biomass measurement and heavy-metal concentrations analysis of plant, pond soil, and

water

Following the procedure of Frérot *et al.* (2006), we carefully washed the collected plants with deionized water to remove the soil particles. The washed plants were separated into aboveground parts (including leaves and stems), rhizomes, and roots, and dried at 80°C for 48 h. The dried parts were weighed and ground. The ground materials were pyrolyzed in conc. HNO_3. Heavy metals (Cu, Zn, and Pb) in the plant tissues were then quantified by inductively coupled plasma atomic emission spectroscopy (ICP-AES; model 757v, Nippon Jarrell-Ash, Kyoto, Japan).

After the soil was air dried at room temperature and passed through sieve (< 2 mm), heavy metals in the soil were also quantified by ICP-AES after digestion in conc. HNO_3-$HClO_3$, and heavy metals in the water were also quantified by ICP-AES. The detection limits for Cu, Zn, and Pb were 5.4, 1.8, and 42 ppb, respectively. Results of four repetitions were averaged, and standard errors were calculated.

Isolation of bacteria from roots

We focus on the bacterial endophyte because arbuscular mycorrhiza and the other fungal structures were rarely observed in the roots stained with trypan-blue. The bacteria were isolated from the roots of *C. virosa* plants harvested in August 2007 by means of the sterilized procedure described by Hata (1997). The roots were carefully washed with running tap water and deionized water, then their surfaces were sterilized in 70% ethyl alcohol for 1 min, 7.5% hydrogen peroxide solution for 3 min, and 70% ethyl alcohol for 1 min. Thereafter, they were rinsed with sterile-deionized water to remove the chemical reagents. The sterilized roots were cut into 10-mm pieces with a sterile scalpel: 60 root pieces were cut from each of individual, and totally 240 pieces

were cut from 4 individuals). We randomly placed 120 root pieces on 1% yeast glucose agar medium (1% YGA) and 120 ones on 1% nutrient broth agar medium (1% NBA). The pieces were incubated at 23°C for 20 days in the dark. All bacteria emerging from the root pieces were purified on each medium.

Zn-solubilization test using bacterial metabolites

Three bacterial discs (5.5 mm diam.) grown on 1% YGA or 1% NBA were used to inoculate 30-ml portions of rhizosphere medium (RSM) (Buyer *et al.* 1989). The inoculated media were incubated with shaking at 150 rpm at 23°C for 2 days in the dark (Incubator SI-600R, AS-ONE, Osaka, Japan). After the incubation, the bacterial cultures were centrifuged at 20,000 x g for 1 h (Hi-mac CR22E, Hitachi, Tokyo, Japan). The supernatants were filtered through a 0.4-μm glass filter (Advantec, Tokyo, Japan) and then through a 0.2-μm PTFE filter (Advantec, Tokyo, Japan). The filtrate pH was measured by pH meter (pH meter F-22, Horiba, Tokyo, Japan).

The pond soil used for the Zn-solubilization test was collected in August 2007. The soils (total amount, 2.5 l) containing no plant litters were randomly collected from 20 points, mixed, air-dried on a plastic bat, and passed through a sieve (< 2 mm). According to the method of Whiting *et al.* (2001), each filtrate (5 ml) was mixed with the dried pond soil (1 g). As a control, RSM alone was mixed with the pond soil. The mixtures were shaken at 150 rpm at 23°C for 2 h to solubilize the heavy metals and then suction-filtered through a 0.2-μm PTFE filter. The filtrates were analysed by ICP-AES. The ratios of Zn amount in the bacterial filtrates to that in RSM filtrates were calculated. When the ratio was over 2, we considered that a certain bacterial strain showed a Zn-solubilization activity. Three replicates were used for the each treatments.

Bacteria detection

Endophyte strains 2-6-2 and 1-4-2, which showed highest Zn-solubilization activity, were determined the gene by 16S rRNA (Lane 1991) . 16S rRNA genes of the strains were amplified by polymerase chain reaction (PCR). The nucleotide primers were eubacterium-specific 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and modified 1494R (5′-TGACTGACTGAGGYTACCTTGTTACGACTT-3′). Sequencing reactions were carried out using an ABI PRISM® BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. The products were then analyzed using an ABI PRISM® 377 DNA Sequencer (Applied Biosystems). Sequences were compared with the DDBJ database using the BLAST search program (National Institute of Genetics, Shizuoka, Japan). Sequences were then aligned using the CLUSTAL X Version 1.83) (EMBL-EBI, Hinxton, UK), and the phylogenetic tree was constructed using the neighbor-joining method. According to the DNA Data Bank of Japan for the 16S rRNA sequences, we identified 2-6-2 as *Pseudomonas putida* AM 184223 and 1-4-2 as *Rhodopseudomonas* sp. AJ 968691 (based on >99% similarity of 16S rRNA sequence).

Inoculation test

Preparation of C. virosa *seedlings and soil sterilization*

Cicuta virosa seeds collected at Chiebun Pond in the city of Nayoro (Hokkaido; 44°45′N, 142°40′E) were obtained from the National Institute of Biomedical Innovation, Japan. These seeds were soaked in 15% hydrogen peroxide solution at 23°C with shaking at 150 rpm for 6 h and then rinsed with four portions of sterilized deionized

water for 30 min at 23°C. The sterilized seeds were incubated on 1/3 Hoagland agar medium (light: 25°C, 14 h; dark: 20°C, 10 h). The seedlings occurred from these seeds were incubated on new medium for 30 days when they were approximately 20 mm high. We used these seedlings as 'axenic' ones in the inoculation of endophytes.

The pond soil collected at the Hitachi mine was sealed in a plastic bag and sterilized by irradiation twice with intermittent gamma rays at a dose of 30 kGy (total, 60 kGy; Japan Irradiation service, Ibaraki, Japan).

Endophyte inoculation and chemical analysis

Endophytes *Pseudomonas putida* AM 184223 and *Rhodopseudomonas* sp. AJ 968691 were applied for the inoculation test. Three axenic *C. virosa* seedlings were transplanted to each sterilized pot (Agri-pot, Kirin, Tokyo, Japan) containing the sterilized pond soil (20 g) and the sterilized deionized water (17 ml). Each bacterial suspension (1 ml, 10^7 units ml^{-1}; + bacteria) was inoculated to seach seedling rhizosphere. As a control, autoclaved each bacterial suspension (1 ml; control) was used as the inoculant for another 4 pots. Furthermore, a pot without the seedlings was prepared. All pots were incubated for 40 days at the light level of 50.4 ± 0.58 μmol s^{-1} m^{-2} (light/dark = 14h, 25°C/10h, 20°C) (N = 20 ; Model LI-250 light meter, Li-Cor, Lincoln, NE, USA) as a pseudo condition of the growing environment at the Hitachi mine. The sterilized deionized water (20 ml) was added to the pots at 14 and 28 days.

After the incubation, the seedlings and the soil were harvested. The numbers of leaves and stems were counted and the seedling height of aboveground was measured for each seedling. Then, the seedlings were separated into aboveground parts (including leaves and stems) and roots (not forming rhizome), and fresh and dry (80°C, 48 h) masses were

measured. One seedling per pot was surface-sterilized as described above and each endophyte was re-isolated on 1% NBA or 1% YGA to confirm Koch's postulate.

After drying at 80°C for 48 h, dried aboveground parts (10 mg) and root (2 mg) were placed in the separate test tubes and then pyrolysed in conc HNO_3 (1 ml) at 130°C in a Dry Thermo Unit (DTU-2C, Taitec, Saitama, Japan). Heavy metals (Cu, Zn, and Pb) and nutrient elements (P, Mg, K, Ca, and Fe) in the pyrolysed solutions were quantified by ICP-AES. Two seedlings per pot were pooled for pyrolysis, because the mass from one seedling was not sufficient for ICP-AES analysis. Four replicates were used for the each treatments.

According to the procedure described by the Committee of Soil Environment Analysis (1997), exchangeable Cu and Zn were extracted from dried soil (3 g) with 0.05 M $Ca(NO_3)_2$ (30 ml) by shaking at 150 rpm at 30°C for 24 h. Exchangeable Pb was extracted from dried soil (3 g) with 1 M ammonium acetate (30 ml, pH 4.5) by shaking at 150 rpm at 30°C for 1 h. We determined the soil pH (H_2O) by mixing the soil with deionized water in a 1:4 ratio. Four replicates were used for the each treatments.

Analysis of indole-3-acetic acid production

Indole-3-acetic acid (IAA), one of phytohormones produced by the endophytes, contributes to the growth and the developments of defense system to heavy metal in plants (Ma *et al.*, 2011). IAA production by *P. putida* and *Rhodopseudomonas* sp. was analysed according to the procedure of Gordon and Weber (1951); absorbance at 530 nm was measured with a UV-visible spectrometer (UV-2450, Shimadzu, Kyoto, Japan). For each strain, data for three replicates were averaged.

Statistical analyses

The numbers of leaves and stems, the seedling heights, the fresh and dry masses of aboveground parts and root, the concentrations of heavy metals and nutrient elements, and the amounts of heavy metals per seedling obtained in the inoculation test were analysed by Smirnov-Grubbs' outlier test, and the data regarded as outliers were omitted. Then, one-factor ANOVA and Scheffé test using PASW Statistics software (v. 8 for Windows, IBM, Armonk, NY, USA) were used to compare the data among treatments.

Results

Heavy metal concentrations in plant, soil, and water at the Hitachi mine

Cu, Pb, and Zn concentrations in the pond soil were high and the drastic changes in these concentrations were not obvious throughout the year (Cu: 760-993 μg g^{-1}; Zn: 273-454 μg g^{-1}; Pb: 670-798 μg g^{-1}). Heavy metal concentrations in the water were below the detection limit (Cu; 5.4, Zn; 1.8, and Pb; 42 ppb,) for ICP-AES analysis.

Aboveground parts biomass was highest in August, but it decreased in September 2006 (Fig. 1a) because of phytophagous insect attack, which had been observed in every summer for the past 5 years in our study site. The aboveground parts biomass did not significantly increase from October 2006 to March 2007.

Of the three metals (Zn, Cu and Pb), Zn showed the highest concentrations in all the plant tissues (Fig. 1b, c, and d). The Zn concentration was much higher in the roots than in the aboveground parts and rhizomes, and that in the roots was the highest in August 2006 (Fig. 1d).

Solubilization of Zn from the pond soil by bacterial metabolites

We isolated 30 strains from the 120 root pieces incubated on 1% NBA, and 24 strains from the 120 root pieces incubated on 1% YGA. 37% (20/54) of these endophytes showed a Zn-solubilizing activity for the mine pond soil. Among the endophytes isolated on 1% NBA, *P. putida* (strain 2-6-2) showed the highest Zn-solubilization ratio (4.91). Among the endophytes isolated on 1% YGA, a *Rhodopseudomonas* sp. (strain 1-4-2) showed the highest solubilization ratio (2.13).

The pH of the RSM in the absence of the bacteria was 6.76 ± 0.04, whereas the pH of the bacterial filtrates varied between 5.02 and 7.37. The pH of the filtrates of 2-6-2 and 1-4-2 were 5.62 and 5.02, respectively.

Inoculation of seedlings with endophytic bacteria

The fresh and dry masses of aboveground parts and roots, the numbers of leaf, the numbers of stem, and the seedling heights of plants inoculated with *P. putida* were significantly greater than those of the control ($P < 0.05$; Table 1, Fig. 2a). The seedlings grown in the absence of *P. putida* showed the chlorosis that is a typical symptom of heavy metal toxicity. The concentration of K in the aboveground parts and the concentration of Mg in the roots of the inoculated seedlings were significantly higher than those of the control seedlings ($P < 0.05$; Table 2). IAA analysis detected at a level of IAA production by *P. putida* (1.53 mg l^{-1} · volume of cultured medium). Re-isolation of each endophyte was successful and Koch's postulate was satisfied in our inoculation test.

Pseudomonas putida inoculation significantly decreased Pb concentration in the aboveground parts ($P < 0.05$; Fig. 3a). On the other hand, heavy metal (Cu, Zn and Pb) concentrations in the roots of *P. putida*-inoculated seedlings did not significantly differ

to those of the control seedlings ($P > 0.05$; Fig. 3b). In contrast, Cu, Zn and Pb amounts of roots (contents per whole roots of a seedling) of *P. putida*-inoculated seedlings were significantly higher than those of the control seedlings ($P < 0.05$; Fig. 4b). Analysis of the soil after *P. putida* inoculation revealed that the values of the exchangeable Zn and Cu were higher in the soil inoculated with *P. putida* than in the soil of control ($P < 0.05$; Table 3). The soil pH was not changed significantly by *P. putida* inoculation ($P > 0.05$; Table 3).

The seedlings inoculated with *Rhodopseudomonas* sp. had significantly greater numbers of leaves, numbers of stems, and fresh and dry masses of roots than the control seedlings ($P < 0.05$; Table 1, Fig. 2b). The seedlings grown in the absence of the *Rhodopseudomonas* sp. (control) showed a chlorosis symptom but the nutrient (P, Mg, K, Ca and Fe) concentrations in the aboveground parts and roots of these seedlings were not significantly different to the inoculated seedlings ($P > 0.05$; Table 2). IAA analysis showed a high level of IAA production by *Rhodopseudomonas* sp. (32.5 mg l^{-1} · volume of cultured medium). Heavy metal concentrations in the seedlings inoculated with *Rhodopseudomonas* sp. and the control seedlings were not significantly different as well ($P > 0.05$; Fig. 3). However, *Rhodopseudomonas* sp. inoculation significantly increased the amount of Pb per roots ($P < 0.05$). This inoculation tended to increase Zn amount per roots, though that was not significant ($P = 0.092$; Fig. 4b). The exchangeable Zn in the soil was significantly increased by *Rhodopseudomonas* sp. inoculation ($P < 0.05$; Table 3). The soil pH was not changed significantly by *Rhodopseudomonas* sp. inoculation ($P > 0.05$; Table 3).

Discussion

Cicuta virosa plants growing in a pond at the Hitachi mine, which is subjected to Cu, Zn and Pb, accumulated high concentrations of these heavy metals (Fig. 1), especially in their roots. The concentrations in the roots are toxic levels in the agricultural crops; Cu concentrations of 2-20 mg kg^{-1}, Zn concentrations of 100-400 mg kg^{-1}, and Pb concentration of 30-100 mg kg^{-1} (plant tissues toxicity limit; Mendez and Maier, 2008; and Broadly *et al.* 2012). Therefore, the fact that *C. virosa* plants were able to survive in the mine pond indicates that this species have a tolerance in the high concentrations of heavy metals more than 20 times compare to crops.

Our results show that 37% (20/54) of the endophytes infected to the *C. virosa* plants solubilized Zn from the pond soil. The endophytes can solubilize Zn mainly by following two ways, 1) producing metal-chelating compounds that bind Zn and 2) decreasing soil pH: immobilized heavy metals in the soil generally become mobile at pH < 5.0 (Larcher 2003). Because the pH of all the bacterial culture filtrates was >5.0, we concluded that the Zn-chelating compounds produced by bacteria influenced Zn solubilization in the present study. Some bacteria produce iron-chelating chemicals called siderophores, some of which can also bind other heavy metals in soil (Miethke and Marahiel 2007). Members of the *Pseudomonas* genus are particularly well known as producers of siderophores (Cornelis and Matthijs 2002; Cornelis and Matthijs 2007; Baysse *et al.* 2000; Visca *et al.* 1992; and Sebat *et al.* 2001). In contrast, there have been few reports on the siderophores of *Rhodopseudomonas* spp.. Moody & Dailey (1984) pointed out that *R. sphaeroides* produced only a minute amount of siderophores. In our inoculation test, without the declines of the pH in cultures, *P. putida* increased the exchangeable Zn and Cu, and also *Rhodopseudomonas* sp. increased the exchangeable Zn (Table 3). Increases in the exchangeable Zn mean that Zn-bioavailability in the soils

inoculated with bacteria was greater than that in the soils without bacteria. *Pseudomonas putida* inoculation significantly increased not only the amounts of Zn, but also those of Cu and Pb per seedling (Fig. 4). These results support our hypothesis that endophytes solubilize Zn *via* bacterial metabolites such as metal-chelating compounds and thus enhance Zn uptake in *C. virosa.*

Our inoculation tests using *P. putida* and *Rhodopseudomonas* sp. clarified that *C. virosa* seedlings grown in the heavy-metal contained soil cannot grow successfully in the absence of these endophytes (see Fig. 2). The concentrations of heavy metals detected in *C. virosa* seedlings without bacterial inoculation would be toxic (Fig. 3). However, these concentrations in the living roots of seedlings inoculated with *P. putida* and *Rhodopseudomonas* sp. were not significantly different from the concentrations in the seedlings without bacteria. These results suggest that the endophytes detoxified the heavy metals in the root tissues. Plants generally detoxify heavy metals by excluding them from plant tissues or by forming the complexes with heavy metals and then accumulating these complexes in their vacuoles (Hall 2002). The metal-chelating compounds produced by *P. putida* and *Rhodopseudomonas* sp. may seep not only into the rhizosphere but also into the plant tissues to detoxify the heavy metals.

Pseudomonas putida and *Rhodopseudomonas* sp. act as plant-growth-promoting rhizobacteria (Patten and Glick 2002; Lee *et al.* 2008), which facilitate the growth of plants in various ways: not only by synthesizing siderophores but also by fixing nitrogen, and phytohormones, solubilizing minerals, and synthesizing low-molecular-mass compounds and enzymes (Glick 1995; Glick 2010; Ma *et al.* 2011). In supporting these views, *Pseudomonas putida* and *Rhodopseudomonas* sp. greatly enhanced the growth of *C. virosa* seedlings even in the presence of the heavy metals.

Increased potassium uptake in the aboveground parts in the presence of *P. putida* and a possible IAA production by both strains seems to contribute to the seedling growth. A dilution of heavy metals in the plant tissues resulting from a rapid plant growth should also decrease the toxicity of heavy metals. When the plants grow extremely fast, the concentrations of inorganic nutrients in the plants decrease (dilution effect; Larcher 2003). Such dilution effect would allow the seedlings to tolerate heavy metals, even if the amount of heavy metals per seedling was increased by bacterial metabolites.

Why could *C. virosa* accumulate high concentration of Zn that generally had a toxicity to many plant species? From an ecological perspective, we point out that heavy-metal accumulation play a role to alleviate the attacks of pathogens and herbivores to *C. virosa* plants as “elemental defence”. The elemental defence is a mechanism to reduce their attacks *via* a high concentration of heavy metals in the plant tissues (Boyd & Martens 1992; Fones *et al.* 2010). In the present study, we did not observe any symptoms of root diseases caused by pathogens, and this suggests that the high concentration of Zn found in the roots may inhibit pathogenic infections in *C. virosa* plants. However, further research is needed to clarify whether the accumulation of heavy metals is an adaptive significance in *C. virosa* or not.

Additionally, in the inoculation test, *C. virosa* seeds collected at Nayoro were used instead of Hitachi mine, because mature seeds could not be collected at our study site. Thus, it is necessary to clarify the interspecific interaction between *C. virosa* and endophytes, considering regional differences. On the other hands, there are possibilities that endophytes originated from Hitachi mine could facilitate heavy-metal tolerance of *C. virosa* with no relation to differences of growing environments, experimental conditions, gene types,

and ecotypes. These results might be important suggestions to clarify specific interactions between endophytes and plants.

Conclusion

The endophytes *P. putida* and *Rhodopseudomonas* sp. facilitated heavy-metal accumulations in *C. virosa* seedlings and enhanced their growth by solubilizing the metals from the soil and by possible production of IAA. Production of metal-chelating compounds that detoxify the heavy metals in the plant tissues and the decreasing in heavy metal concentration of plants *via* the rapid plant growth seemed to contribute the heavy-metal tolerance of *C. virosa*. Further investigations are needed to identify metal-chelating compounds produced by bacterial strains, and their roles in the metal-detoxification and the metal-solubilization in the soil.

Banks of buried seeds and/or seedlings (juvenile plants) of *C. virosa* play important roles to regenerate the population (see also Ajima *et al.* 1999; Tsuda & Kikuchi 1993). From our examinations, we suggest that the endophytes can assist the establishment and persistence of *C. virosa* seedlings. In the disturbed and polluted sites such as mines, the managements and conservations with respect to the plant-bacterial interactions must be noted to restore and remediate the aquatic ecosystems. The effects of endophytes on other stage of *C. virosa* plants also should be examined in the future.

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Figure legends

Fig. 1. Biomass and heavy metal concentrations of *C. virosa* at the study site. (a) Biomass of aboveground parts, rhizomes and roots. (b) Heavy metal concentrations in aboveground parts. (c) Heavy metal concentrations in rhizomes. (d) Heavy metal concentrations in roots. Rhizomes were not formed from October to April.

Fig. 2 Seedlings grown in the inoculation test. (a) Seedlings grown for 40 days after the inoculation of *P. putida*. (b) Seedlings grown for 40 days after the inoculation of *Rhodopseudomonas* sp.. The size bars represent 10 mm.

Fig. 3 Heavy metal concentrations of *C. virosa* grown in the inoculation test. (a) Heavy metal concentrations in aboveground parts of seedlings grown for 40 days after the inoculation. aboveground parts include aboveground parts including leaves and stems. (b) Heavy metal concentrations in roots of seedlings grown for 40 days after the inoculation. Different letters indicate statistically significant difference among treatments in ANOVA-comparisons and post hoc Scheffé test with $P < 0.05$.

Fig. 4. Heavy metal amounts of *C. virosa* grown in the inoculation test. (a) Amount of heavy metals in aboveground parts per seedling grown for 40 days after the inoculation. (b) Amount of heavy metals in roots per seedling grown for 40 days after the inoculation. Different letters indicate statistically significant difference among treatments in ANOVA-comparisons and post hoc Scheffé test with $P < 0.05$.

Table 1 *Cicuta virosa* growth in the inoculation test

	Inoculation condition		
	Control	*P. putida*	*Rhodopseudomonas* sp.
Number of leaves	6 ± 1^{c}	13 ± 1^{a}	9 ± 1^{b}
Number of stems	2 ± 0^{b}	4 ± 0^{a}	3 ± 0^{a}
Seedling height (mm)	24.1 ± 2.2^{b}	62.9 ± 6.1^{a}	37.2 ± 3.8^{b}
Fresh mass of aboveground parts (mg)	25.7 ± 2.5^{b}	73.4 ± 11.5^{a}	46.7 ± 7.14^{a}
Fresh mass of roots (mg)	8.18 ± 1.79^{b}	52.6 ± 9.40^{a}	31.6 ± 3.29^{a}
Dry mass of aboveground parts (mg)	3.03 ± 0.36^{b}	10.3 ± 1.95^{a}	5.94 ± 1.30^{b}
Dry mass of roots (mg)	0.96 ± 0.22^{b}	5.92 ± 1.00^{a}	3.88 ± 1.06^{a}

Different letters indicate statistically significant difference among treatments in ANOVA-comparisons and post hoc Scheffé test with $P < 0.05$. n = 4.

Table 2 Nutrient concentrations of *C. virosa* in the inoculation test. (A) Shoots. (B) Roots.

(A)

	Inoculation condition		
mg g^{-1}	Control	*P. putida*	*Rhodopseudomonas* sp.
P	19.5 ± 1.93^{b}	10.5 ± 1.08^{a}	13.8 ± 1.76^{ab}
Mg	2.88 ± 0.18^{a}	2.65 ± 0.24^{a}	2.69 ± 0.20^{a}
K	4.48 ± 0.44^{b}	24.8 ± 2.03^{a}	11.6 ± 3.99^{b}
Ca	9.78 ± 1.85^{a}	8.33 ± 0.81^{a}	7.54 ± 1.37^{a}
Fe	0.47 ± 0.11^{a}	0.14 ± 0.03^{a}	0.18 ± 0.06^{a}

(B)

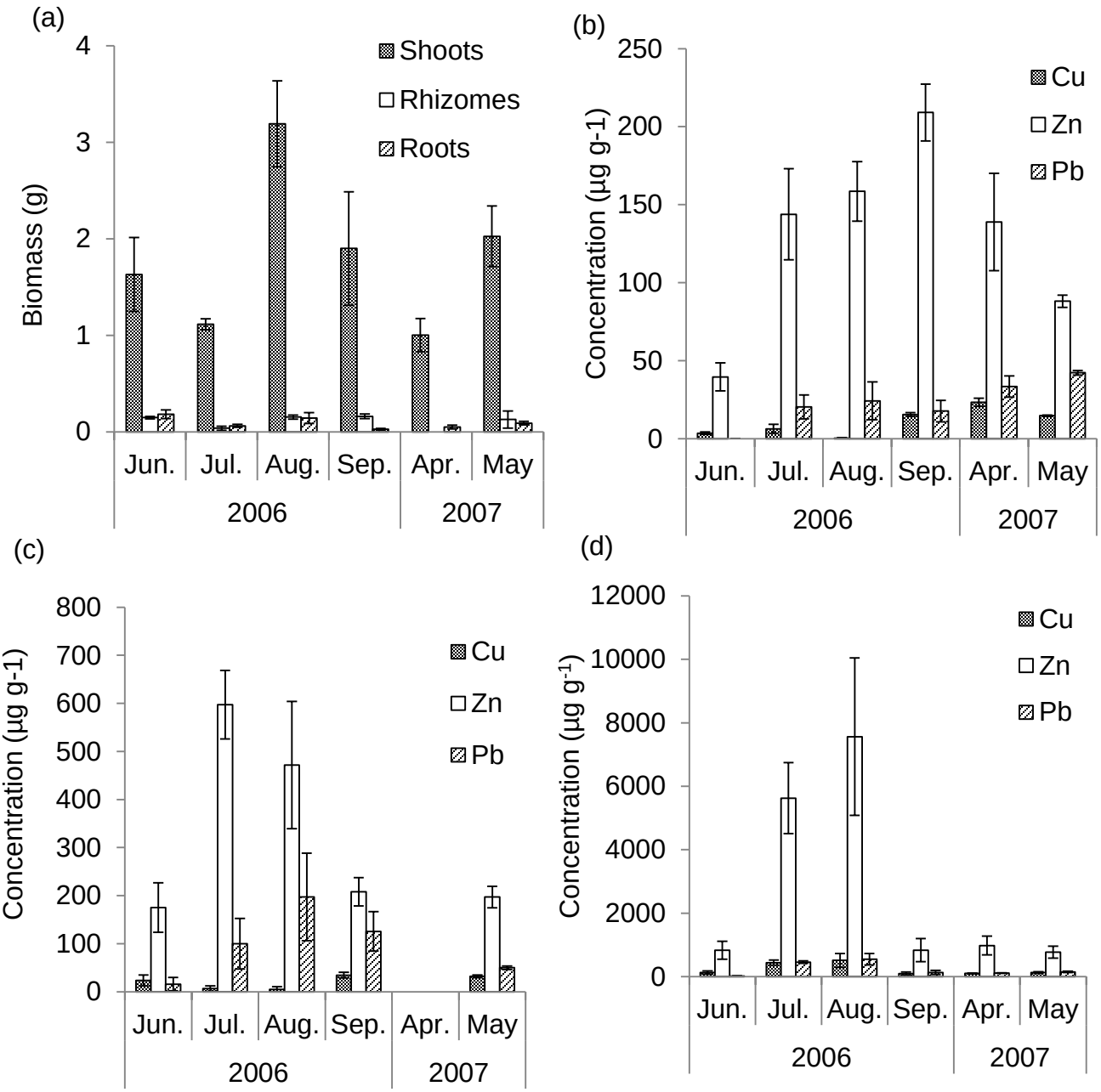
	Inoculation condition		
mg g^{-1}	Control	*P. putida*	*Rhodopseudomonas* sp.
P	14.6 ± 0.61^{a}	11.4 ± 0.40^{a}	14.85 ± 1.71^{a}
Mg	2.13 ± 0.15^{b}	3.60 ± 0.18^{a}	2.70 ± 0.17^{b}
K	11.2 ± 1.93^{a}	19.0 ± 3.48^{a}	17.0 ± 5.62^{a}
Ca	15.1 ± 2.92^{a}	6.21 ± 0.68^{a}	8.42 ± 1.03^{a}
Fe	1.81 ± 0.35^{a}	1.27 ± 0.20^{a}	0.84 ± 0.10^{a}

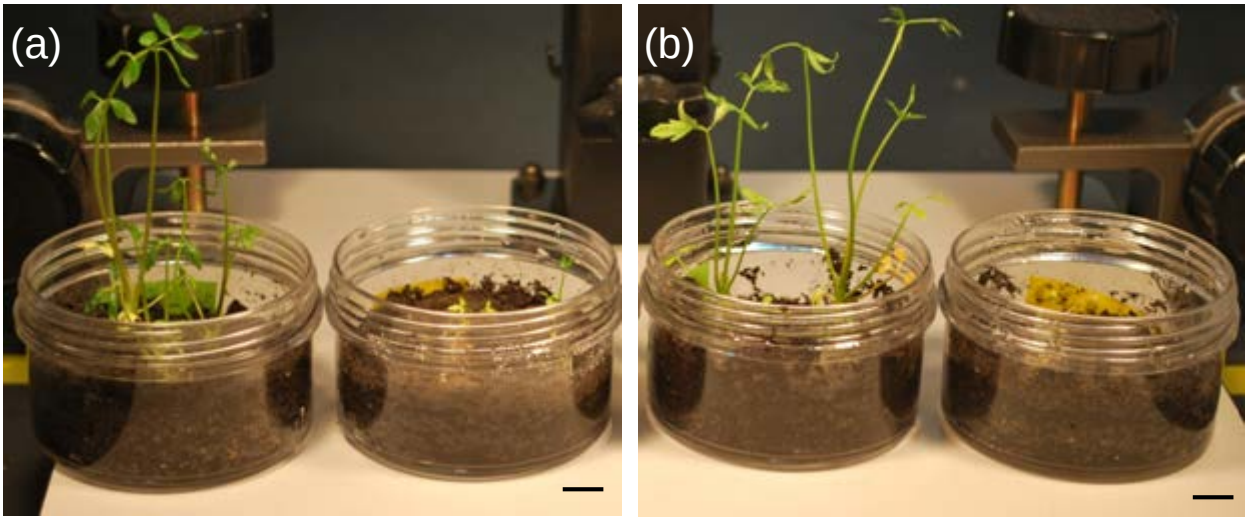
Different letters indicate statistically significant difference among treatments in ANOVA-comparisons and post hoc Scheffé test with $P < 0.05$. n = 4.

Table 3 Exchangeable heavy metals and pH in pond soil after the inoculation test

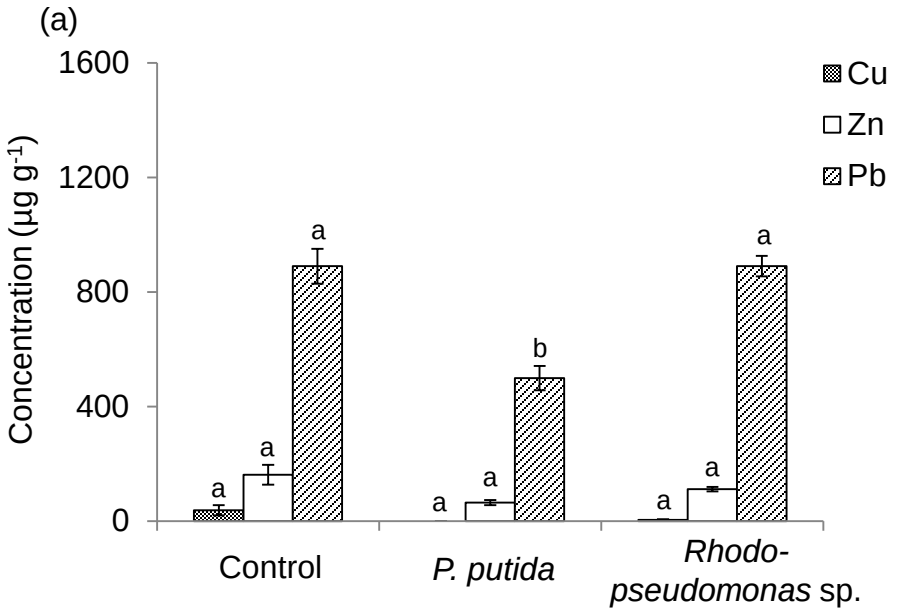
Exchangeable heavy metals ($\mu g\ g^{-1}$) and pH	Inoculation condition		
	Control	*P. putida*	*Rhodopseudomonas* sp.
Cu	2.45 ± 0.07^{b}	2.88 ± 0.08^{a}	2.53 ± 0.09^{ab}
Zn	29.5 ± 0.43^{b}	34.6 ± 0.56^{a}	33.2 ± 1.06^{a}
Pb	128 ± 4.56^{a}	145 ± 4.52^{a}	141 ± 7.37^{a}
pH	5.60 ± 0.03^{a}	5.52 ± 0.01^{a}	5.61 ± 0.01^{a}

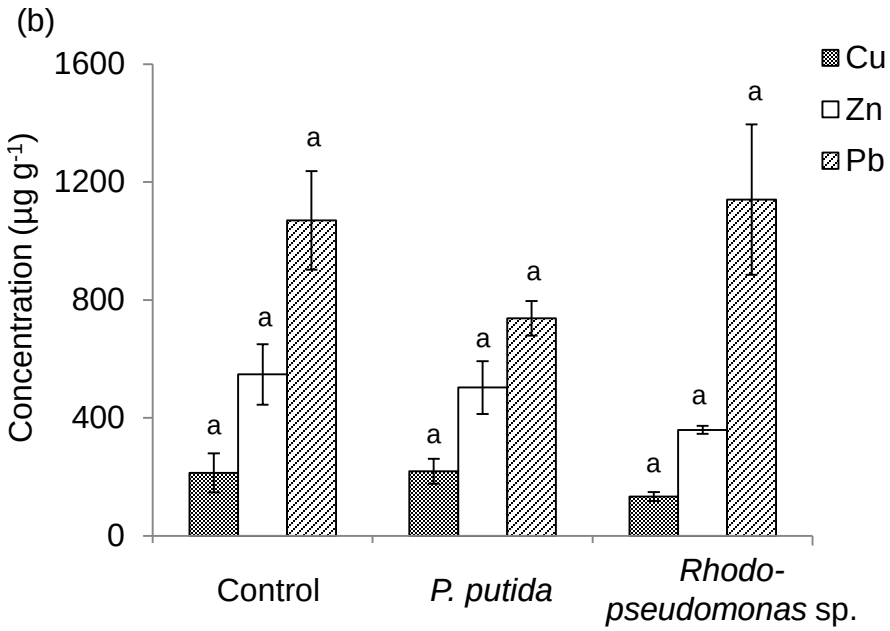
Different letters indicate statistically significant difference among treatments in ANOVA-comparisons and post hoc Scheffé test with $P < 0.05$. n = 4.

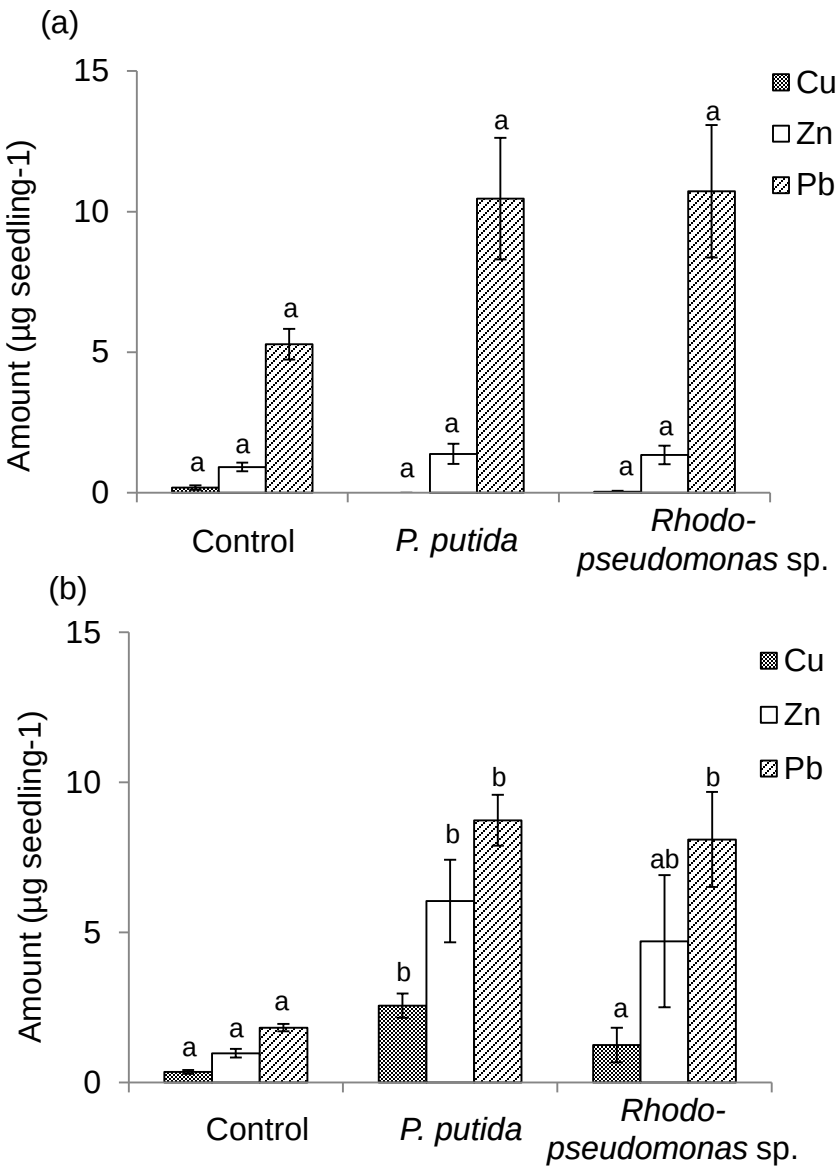

(a)
Biomass (g)
0
1
2
3
4
Shoots
Rhizomes
Roots
Jun.
Jul.
Aug.
Sep.
Apr.
May
2006
2007
(b)
Concentration (µg g-1)
0
50
100
150
200
250
Cu
Zn
Pb
Jun.
Jul.
Aug.
Sep.
Apr.
May
2006
2007
(c)
Concentration (µg g-1)
0
100
200
300
400
500
600
700
800
Cu
Zn
Pb
Jun.
Jul.
Aug.
Sep.
Apr.
May
2006
2007
(d)
Concentration (µg g-1)
0
2000
4000
6000
8000
10000
12000
Cu
Zn
Pb
Jun.
Jul.
Aug.
Sep.
Apr.
May
2006
2007



+ bacteria control + bacteria control


(a)
1600
1200
800
400
0
Concentration (µg g-1)
Cu
Zn
Pb
a
a
a
a
a
b
a
a
a
Control
P. putida
Rhodo-pseudomonas sp.


(b)
1600
1200
800
400
0
Concentration (µg g-1)
Cu
Zn
Pb
a
a
a
a
a
a
a
a
a
Control
P. putida
Rhodo-pseudomonas sp.


(a)
15
10
5
0
Amount (µg seedling-1)
Cu
Zn
Pb
a
a
a
a
a
a
a
a
a
Control
P. putida
Rhodo-pseudomonas sp.
(b)
15
10
5
0
Amount (µg seedling-1)
Cu
Zn
Pb
a
a
a
b
b
b
a
ab
b
Control
P. putida
Rhodo-pseudomonas sp.