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Infection, colonization and growth-promoting effects of tea plant (*Camellia sinensis* L.) by the endophytic bacterium *Herbaspirillum* sp. WT00C

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The endophytic *Herbaspirillum* sp. WT00C, isolated from tea plant, seems to have a potential ability to promote tea-plant rooting and budding due to its capability of producing indole-3-acetic acid (IAA), ammonia and siderophores. Thus, the present study was aimed to verify whether this bacterium could be used for tea cultivation as an environment-friendly bioaccelerator. To evaluate its potential use in promoting tea-plant rooting and bud growth, *Herbaspirillum* sp. WT00C was characterized using several methods. Observation by bacterial infection found that the bacterium only went into plants via plant vulnus when irrigation, sprinkling and traumatic infection were applied. Whatever irrigation, sprinkling or traumatic infection was applied, all tea plant, vegetables, rice and wheat tested in this study did not show any growth inhibition or disease symptom. Observation by bacterial count test also found that the bacterium colonized only in tea plant, but not in vegetables, rice or wheat. To test the effect of *Herbaspirillum* sp.WT00C on tea-cutting rooting and budding, tea cuttings were soaked with the diluted bacterial culture. Observation at 280 day postinoculation found that the tea-seedling rate approached to 100%, and average newborn shoot length and lateral root number of tea seedlings increased 88% compared to control groups. In addition, the bacterium was found only in those tea cuttings treated with the bacterium, but not in their newborn shoots and leaves. Inoculating the bacterium to the upper incision of tea twigs in the field also enhanced the growth of newborn shoots. Our studies demonstrated that *Herbaspirillum* sp.WT00C was a tea-specific endophyte with the ability to stimulate the lateral root formation and bud growth of tea cuttings, and paved the way for its application in propagation of tea cuttage as a novel bioaccelerator.

Key words: Endophytic bacterium, bioaccelerator, tea cottage, traumatic infection, adventitious root formation.

INTRODUCTION

Herbaspirillum sp. WT00C, isolated from *Camellia sinensis* L., was classified as a novel member in the genus *Herbaspirillum* based on its physiochemical characteristics and 16S ribosomal DNA (rDNA) sequence (Wang et al.

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2014). Different from *Herbaspirillum seropedicae*, *Herbaspirillum frisingense* and *Herbaspirillum lusitanum* able to fix nitrogen (Baldani et al., 1986; Kirchhof et al., 2001; Valverde et al., 2003), *Herbaspirillum* sp. WT00C did not exhibit any nitrogen-fixing activity. However, this endophytic bacterium was able to produce indole-3-acetic acid (IAA), ammonia and siderophores (Wang et al., 2014). IAA, ammonia and siderophores have been thought to play a role in inducing adventitious root formation in dicots or monocots and improving plant growth and development (Glick 2012; Phillips et al., 2011; McSteen, 2010; Dubis et al., 2003; Temple et al., 1998; Neilands, 1995; Barton and Hemming, 1993). In view of its physiochemical properties, *Herbaspirillum* sp. WT00C may perhaps be useful in tea cultivation.

C. sinensis (L.) is native to East, South and Southeast Asia, but it is today cultivated across the world in tropical and subtropical regions. Two principal varieties, the small-leaved Chinese variety plant (*C. sinensis var. sinensis*) and the large-leaved Assamese plant (*C. sinensis var. assamica*), are mainly used for production of white tea, yellow tea, green tea, black tea or twig tea. Although *C. sinensis*can perform sexual reproduction, farmers usually propagate them via asexual manner in order to maintain genetic homogeneity of tea offspring. Many strains and clonal varieties used currently in tea gardens have been established with the superior clones selected from native population. Since 1960s, vegetative propagation has widely been used for rapid production of tea seedlings in tea plantations (Choubey et al., 2013). However, a major problem faced by farmers is low seedling rate during vegetative propagation. Raising success rate in tea-cuttage propagation is a crucial issue, especially for those high-quality tea strains. Thus, there is an urgent need for new environmental-friendly techniques.

Although the endophytic *Herbaspirillum* sp. WT00C had a great potential as a bio-accelerator, its efficient route of infection, host-spectrum, pathogenicity, and effectiveness of promoting tea-plant rooting and budding were unclear. In this study, our aims were twofold: first, to investigate the infection mode, host-spectrum and pathogenicity of *Herbaspirillum* sp. WT00C, and second, to examine effectiveness of inducing lateral-root formation and bud growth in order to verify whether the bacterium can be really used as an environment-friendly bioaccelerator for tea cultivation. In our study, we found that *Herbaspirillum* sp. WT00C entered plants via traumatic infection, but did not cause any noticeable disease symptom. The bacterium colonized only in tea plant, other than in other crops. Furthermore, we also showed that *Herbaspirillum* sp. WT00C significantly enhances rooting and bud growth of tea cuttings. These characteristics of *Herbaspirillum* sp. WT00C appear to satisfy basic conditions required for a green bioaccelerator. Our study paves the way for application of the endophytic bacterium *Herbaspirillum* sp. WT00C to raise the success rate in tea cuttage or stimulate bud growth in tea cultivation.

MATERIALS AND METHODS

Bacterial culture

Herbaspirillum sp. WT00C was isolated from *C. sinensis* (L.) and stored in our laboratory. It was routinely cultured in Luria Bertani (LB) medium supplemented with 10 μg/ml spectinomycin and 10 μg/ml ampicillin at 37°C unless otherwise stated. In a large-scale preparation, the stock culture was inoculated into 5 ml LB medium with two specific antibiotics, and incubated at 37°C overnight. Then bacterial culture was transferred into 500 ml fresh LB medium containing the same antibiotics and grown at 37°C until OD_{600} (optical density at 600 nm) of 1.0.

Construction of the *Herbaspirillum* sp. WT00C strain with strong ampicillin resistance

Herbaspirillum sp. WT00C only displayed low resistance (10 μg/ml) against spectinomycin and ampicillin. Such low antibiotic resistance was not suitable for investigating bacterial invasion route and colonization. To enhance the ampicillin resistance of *Herbaspirillum sp*. WT00C, a pUC18 plasmid was introduced into the bacterium via electroporation. Competent cells were made using glycerol according to the standard process (Sambrook et al., 2001). Electroporation was performed on an electroporator (Electroporator 2510, Eppendorf). After electroporation, bacterial cells were incubated in nutrient broth (0.5% peptone, 0.3% beef extract, 0.5% NaCl) at 37°C for 1 h, spread on nutrient agar plates (nutrient broth plus 1.5% agar) containing 10 μg/ml spectinomycin and 100 μg/ml ampicillin, and grown at 37°C for 24 h. Bacterial colonies with ampicillin and spectinomycin resistance were picked out, and further confirmed by checking the pUC18 DNA. The plasmid DNA was extracted from each colony and examined on 10% agarose gel according to the standard method (Sambrook et al., 2001). The strain with strong ampicillin resistance was only used to investigate the route of bacterial invasion or colonization in this study.

IAA color assay

IAA was determined *in vitro* based on the method reported previously (Brick et al., 1991; Holt et al., 1994). Briefly, bacterial culture was inoculated in LB broth containing 10 μg/ml spectinomycin and 10 μg/ml ampicillin at 37°C until OD_{600} of 0.6. Then, 50 μl bacterial culture was inoculated into 5 ml LB medium described above plus 1 μg/ml L-tryptophan. After incubated at 37°C until OD_{600} of 1.0, bacterial cultures were centrifuged at 3000 rpm for 30 min. The supernatant (1 ml) was taken and mixed with 4 ml of Salkowski's reagent (35% perchloric acid, 10 mM $FeCl_3$), and then the mixture was placed in a dark room for 20 min. The color intensity was recorded at 530 nm on a spectrophotometer (Shimadzu UV-2550). All data were collected from three replications.

Test of inoculation modes

Three different methods, irrigation, sprinkling and traumatic infection were used to test how *Herbaspirillum* sp. WT00C invaded tea plants. Tea plants were cultivated in pots located in a greenhouse. All experiments were divided into 4 groups, and each group had 10 tea seedlings. When the height of tea seedlings in pots approached to 15 to 20 cm, equal amount of bacteria (3.02 ×

10^{10}cfu) was poured into pots at tea roots or sprayed over tea leaves. In traumatic infection, a hole on the tea stem at 4 cm above the roots was firstly made using a sterile needle, and then the wound was covered by a piece of cotton wool containing the bacterial culture (2.72×10^{8}cfu). Equal volume of deionized water (dH_2O) was used in each control group. The bacterial dosage was used referring to previous research reports (Berta et al., 2014; Straub et al., 2013; Stefan et al., 2013). After treatments, the roots, leaves and stems including upper stem (2 cm above the inoculation point), lower stem (2 cm below the inoculation point) and inoculation-point zone were collected at predefined intervals, and *Herbaspirillum* sp. WT00C inside different organs of tea seedlings was examined according to the standard protocol used for isolation of an endophytic bacterium. In brief, tea samples were firstly soaked in 75% ethanol for 4 min. washed 3 times with sterile water, and then soaked in 0.1% mercuric chloride for 1 min. Finally, all samples were washed 5 times with sterile water until no bacterium grew in the final wash. For qualitative assay, the sterilized roots, stems or leaves were cut into thin slices (0.5 cm thickness) by a blade, and then thin slices were placed on LB plates containing 10 μg/ml spectinomycin and 100 μg/ml ampicillin. After incubation at 37°C for 48 h, bacterial growth around each thin slice was observed by eye. For quantitative assay, different parts of tea plants were grinded in PBS (phosphate buffer saline) to homogenate in a glass grinder under aseptic condition, and then 200 μl of homogenate was taken to spread LB plates with two antibiotics, and all plates were incubated at 37°C for 48 h. Finally, bacterial colonies were counted. Bacterial content was calculated based on the following formula, in which the colony number was an average obtained from triplicate tests.

$$\text{Number of bacteria per gram of tissue} = \frac{\dfrac{\text{the number of colony-forming units}}{\text{the volume for plating (ml)}} \times \text{total volume of sample (ml)}}{\text{the weight of tissue (g)}}$$

Inoculation test of tea cuttings

The experiment was conducted at the tea farm of Xianning Academy of Agriculture Science. *C. sinensis* cv. Echa 1 was used as parent materials in tea-cuttage propagation. Tea shoots were cut into two parts: tender green-cutting (upper part) and hard red-cutting (lower part). each cutting carried a single node with a leaf and its length was about 5 cm. Tea cuttings including tender green-cutting in group A and hard red-cutting in group B were separately soaked in the bacterial culture diluted by H_2O (2:1) for 1 to 3 h. In the control group, tea cuttings were soaked in water under the same condition. In each group, 200 tea cuttings were used. After treated, all tea cuttings were taken out and dried for a while until no liquid dropped down. Finally, tea cuttings were immediately planted in the nursery, and the field management was done according to the routine method for tea-cuttage propagation. The growth of tea seedlings was observed and recorded at predefined intervals. Data were analyzed via SPSS software, and analysis of variance (ANOVA) gave P values of less than 0.05 in each case.

Inoculation test of tea twigs

To further confirm if the bacterium really stimulates growth of tea buds, tea twigs in the tea garden were pruned with a trimmer, and then 20 μl of the bacterium culture with different dilutions (0.5 to 2:1) was inoculated to each incision on the top of 500 twigs of tea bush. Initial concentration of bacterial cells was 7.14×10^{7} cfu/ml. The content of bacterial cells inoculated in each group was 9.5×10^{5}cfu for 2:1 dilution, 7.14×10^{5}cfu for 1:1 dilution, and 4.76×10^{5}cfu for 1:2 dilution. In the control group, the same amount of LB medium diluted with H_2O (2:1) was used. The field management was done according to the routine method for tea cultivation. The growth of lateral buds of the pruned tea twigs was observed and recorded. The experiment was conducted in a randomized design with five replicates, and each replicate containing 100 samples. Experimental data were analyzed via SPSS software, and analysis of variance gave P values of less than 0.05 in each case.

RESULTS

Invasion of *Herbaspirillum* sp. WT00C into tea plant via traumatic infection

Three different methods, irrigation, sprinkling and traumatic infection were tested to know how *Herbaspirillum* sp. WT00C invaded tea plants. In this study, the *Herbaspirillum* sp. WT00C carrying a pUC18 plasmid was used owing to its strong ampicillin resistance. After tea plants were treated with different processes, the bacteria inside roots, leaves and stems were examined. The results were summarized in Table 1. As expected, the bacterium within tea leaves, stems and roots was not found in control group. In group A and B, the bacterium inside the tea seedlings was also undetectable whether irrigation or sprinkling was applied. Different from group A and B, the bacterium appeared in the stem of tea seedlings in group C when traumatic infection was applied. Moreover, bacterial cells were found to migrate upward and downward inside the stem of tea seedlings (Table 1).

Colonization of *Herbaspirillum sp.* WT00C in other farm crops

Like tea plant, *Brassica campestris, Brassica rapa, Oryza sativa* and *Triticum aestivum*, were also tested. All seedlings of four crops were firstly planted in pots and grown for 30 days in a greenhouse. Then, three methods described above were applied to test if *Herbaspirillum sp*. WT00C infected these plants. After treatment, data were consecutively collected for 40 days. During our experiments, all plants in experimental groups grew as well as those in control groups. Similar to the control group, *Herbaspirillum sp*. WT00C within roots, stems or leaves of four crops was not detectable whether irrigation or sprinkling was applied. In traumatic infection, the bacterium was only found in stems of four plants. The detailed results were summarized in Table 2. In those stems of *B. campestris* and *B. rapa*, the bacterium survived until the 15^{th} day postinoculation, but bacterial numbers decreased progressively by an order of magnitude. After 15 day postinoculation, the bacterium disappeared completely from those stems. In *O. sativa* and *T. aestivum*, the bacterium was detectable until the 5^{th} day postinoculation with a rapid decrease of its number. After 5 days postinoculation, the bacterium was not detectable inside the stems of either rice or wheat.

Table 1. Bacterial distribution in different parts of tea plants treated by different inoculation methods. Group A: irrigation; Group B: sprinkling; Group C: traumatic infection; Control group: no treatment.-: undetectable; +: bacterial growth. In this study, 5-10 thin slices for each part were tested at different posttreatment times. Data were recorded from three replications.

Position		1 day	5 days	11 days	33 days
Group A	Roots	–	–	–	–
	Upper stem	–	–	–	–
	Lower stem	–	–	–	–
	Leaves	–	–	–	–
Group B	Roots	–	–	–	–
	Upper stem	–	–	–	–
	Lower stem	–	–	–	–
	Leaves	–	–	–	–
Group C	Roots	–	–	–	–
	Upper stem	–	+	+	+
	Inoculation point	+	+	+	+
	Lower stem	+	+	+	+
	Leaves	–	–	–	–
Control group	Roots	–	–	–	–
	Upper stem	–	–	–	–
	Lower stem	–	–	–	–
	Leaves	–	–	–	–

Table 2. Bacterial content inside the stems of four crops. After traumatic infection, plant stems were collected from 1 to 40 days and grinded to homogenate. Bacterial numbers were quantitatively counted as described in experimental section. Here, bacterial content was expressed as colony-forming units (cfu) per gram wet weight of plant stems.

Plants	1 day	5 days	10 days	15 days	20 days	30 days	40 days
Brassica campestris	1.71×10^{8}	1.18×10^{4}	1.33×10^{3}	332	0	0	0
Brassica rapa	1.73×10^{8}	5.88×10^{4}	1.04×10^{3}	360	0	0	0
Oryza sativa	1.14×10^{8}	1.83×10^{2}	0	0	0	0	0
Triticum aestivum	1.70×10^{8}	8.15×10^{2}	0	0	0	0	0

Meanwhile, the thin-slice method described above was also used to test which part of the stem in four crops contained the bacterium. At the 4th day of bacterial infection, the bacterium mainly remained around the inoculation point region (data not shown).

Effects of *Herbaspirillum sp.* WT00C in propagation of tea cuttage

After treated with *Herbaspirillum sp.* WT00C for different times, tea cuttings were planted in the tea nursery. The tea-seedling rate in three experimental groups approached to 100%. Figure 1 showed tea seedlings growing for 160 days in the nursery after treatment. Clearly, those tea seedlings in all three experimental groups were in vigorous growth with dark green colors. The number of lateral roots in experimental groups was 26±3, 63 more than that (16±4) for those control groups. Average newborn shoot length and root length were 15±1.4 cm and 9±1.2 cm for experimental groups and 13±1.8 cm and 8±1.8 cm for control groups. In addition, those tea seedlings from the tea-cuttings soaked in bacterial culture for 1, 2 or 3 h did not show significant deference as shown in Figure 1. One-hour treatment could be enough for bacterial inoculation.

Data were also collected at the 280th day post treatment and shown in Figure 2. Although, root length did not show significant difference, newborn shoot length and root number were significantly different between three experimental groups and control groups. As compared to the control groups, average newborn shoot

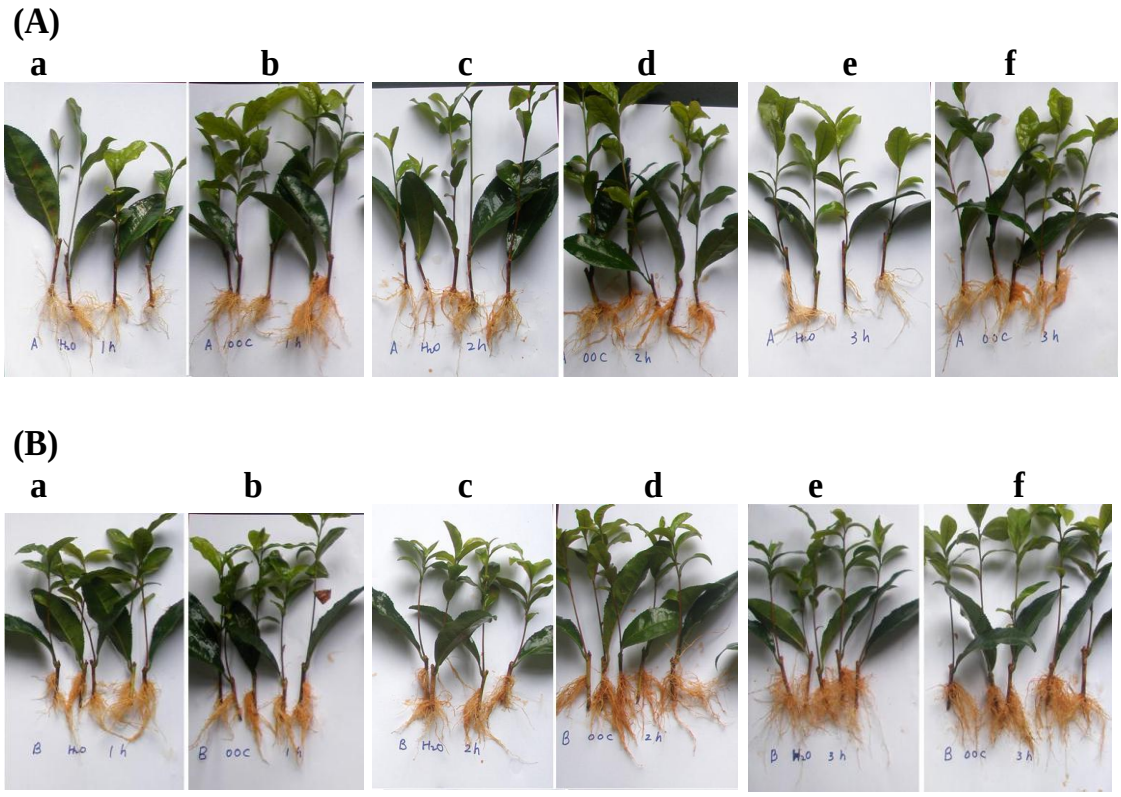

Figure 1. Comparison of tea seedlings between experimental groups and control groups. All seedlings were randomly chosen at the 160th day postcuttage. (A) Seedlings from tender green-cutting; (B) Seedlings from hard red-cutting.a, c, e: tea cuttings soaked in water for 1, 2 and 3 h; b, d, f: tea cuttings soaked in the bacterial culture for 1, 2 and 3 h.

length and lateral root number in the experimental groups increased 87.5%. In addition, effects of *Herbaspirillum sp.* WT00C on tea-seedling growth between the tender green-cutting group and the hard red-cutting group were similar. Data for those tea seedlings derived from those tea-cuttings treated with the bacterial culture for 1, 2 or 3 h also did not show significant difference. The bacterium displayed the same effect on promoting lateral root formation or bud growth whatever hard red cuttings or tender green cuttings were used.

Bacterial distribution in tea seedlings growing for either 160 or 280 days was examined. *Herbaspirillum sp.* WT00C was verified by growing the thin slices from different parts of tea-seedlings on the LB plates, and then further confirmed by IAA color reaction. The bacterium was only found in those tea cuttings treated with the bacterial culture. In newborn shoots, leaves and roots, the bacterium was undetectable.

Effects of *Herbaspirillum* sp. WT00C on promoting growth of lateral buds

To further confirm bacterial effects on promoting growth of tea buds, we pruned tea twigs at the top of tea bush with a trimmer, and then inoculated the bacterium with different dilutions to the incision on the upper part of tea twigs. At the 60th day post treatment, length and weight of lateral buds were measured, and the results were shown in Figure 3. Average length of newborn lateral shoots and weight of 100 lateral shoots in all experimental groups increased 4.6 cm and 18 g respectively as compared to the control group. Bacterial test also showed that *Herbaspirillum sp*. WT00C existed in those pruned old tea twigs, but not in newborn tender shoots (data not shown).

DISCUSSION

In this study, microbiological characteristics of *Herbaspirillum sp.* WT00C were further investigated to verify whether it can be really used as a novel bioaccelerator. Our study found that this endophytic bacterium entered into plants via traumatic infection, but did not infect plants through irrigation or sprinkling. It colonized only in tea plants but not in vegetables, rice and wheat. These results demonstrated the entry of

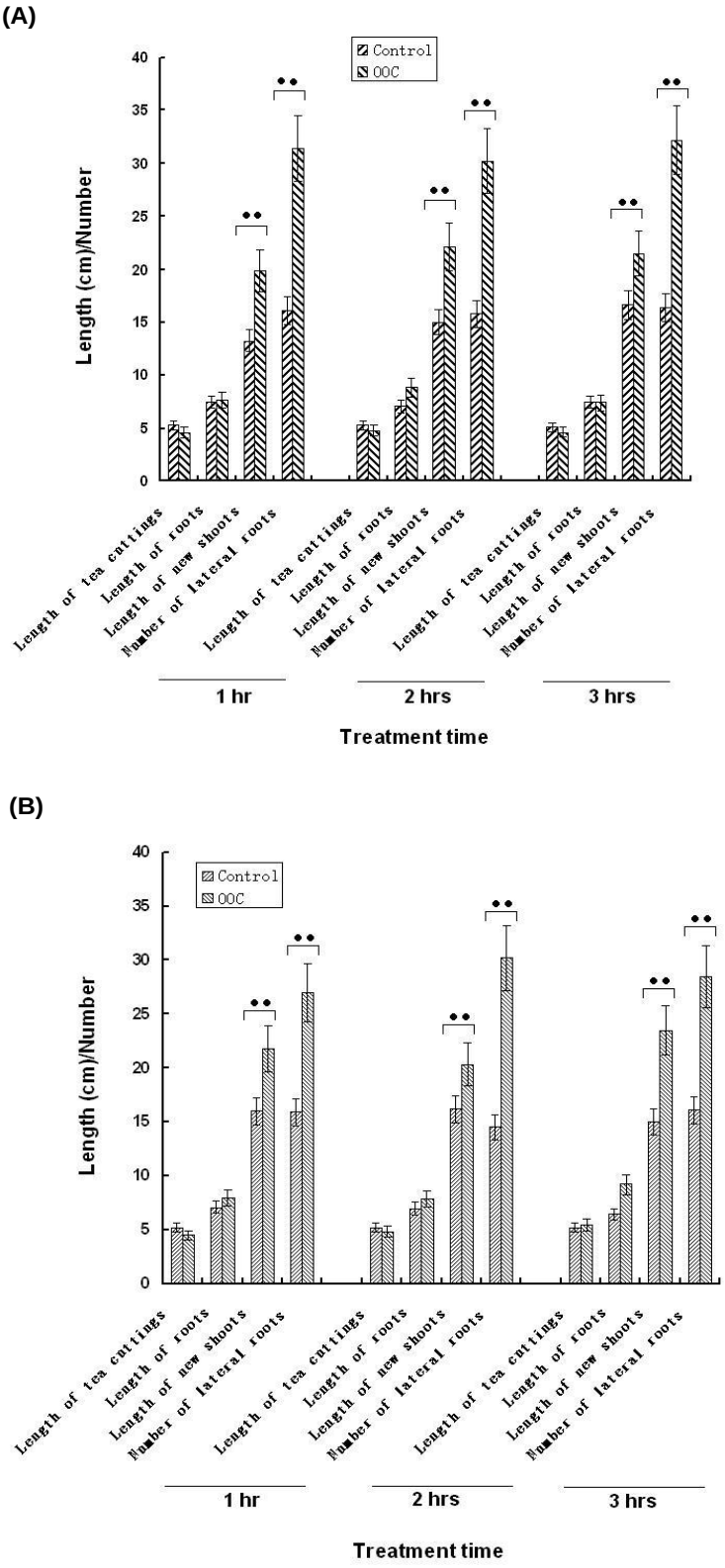

Figure 2. Effects of Herbaspirillum sp. WT00C on the growth of newborn roots and shoots of tea cuttings. The data were collected from 100 tea seedlings in each group, which were randomly chosen in the nursery at the 280th day postcuttage. Significant difference between experimental groups and control groups was labeled. (A) tender green-cutting; (B) hard red-cutting.

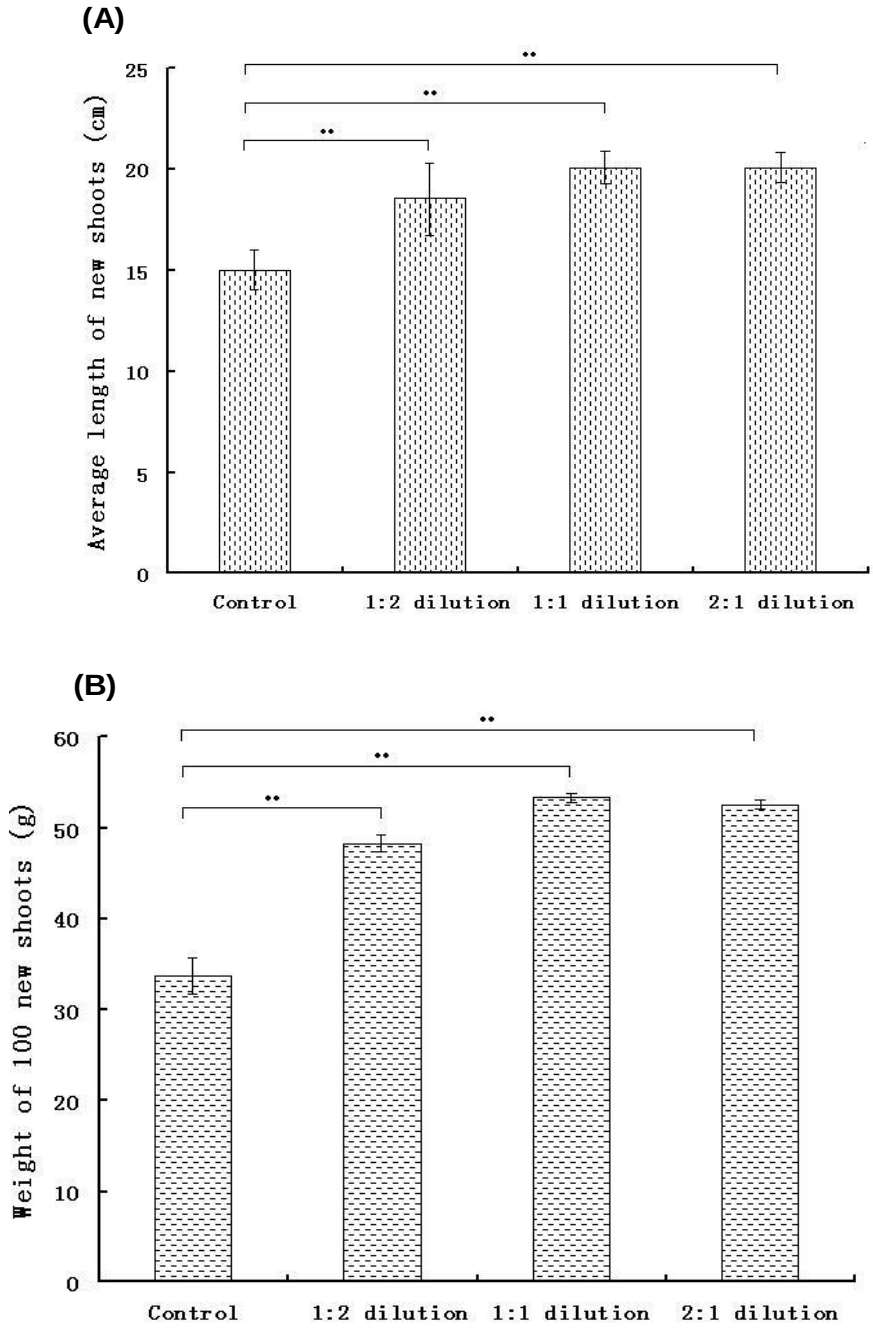

Figure 3. Effects of Herbaspirillum sp. WT00C on the growth of lateral buds of tea twigs. Data were collected from 500 tea buds in each group, which grew 60 days after bacterial inoculation. Significant difference between experimental groups and control groups was labeled. (A) average length of 100 lateral buds; (B) average weight of 100 lateral buds.

Herbaspirillum sp. WT00C into plants via plant vulnus, and suggested that the bacterium might not colonize in either brassicaceous vegetables or gramineous plants although it could survive for a period of time. More importantly, the bacterium did not cause any noticeable disease symptom or growth inhibition in all tests. These characters of the bacterium could satisfy those basic conditions required for a green bioaccelerator. Once it entered into tea plants through incisions at the double ends of tea cuttings or at the top of tea twigs, *Herbaspirillum* sp. WT00C was able to colonize in tea plants and produce IAA, ammonia and siderophores. Bacterial cells of *Herbaspirillum* usually colonized in vascular tissues (James et al., 1997). Our studies clearly

showed that vegetative growth, length of newborn shoots, number of lateral roots in the infection group were superior to those in the uninfected group. Our data suggested that this bacterium indeed had the ability to stimulate lateral root formation and bud growth of tea cuttings. More adventitious root formation was certainly favorable for raising the seedling rate of tea cuttings. In addition, the bacterium was only found in tea cuttings, but not in their newborn shoots and leaves. This finding could rule out the possibility that bacterial existence in newborn shoots or leaves may affect quality characteristics of tea. Accordingly, *Herbaspirillum* sp. WT00C appears to be a good candidate for a bioaccelerator applicable for tea-cuttage propagation in the fields.

Cuttage propagation is a common technique used widely for a rapid production of tea seedlings in tea plantations (Choubey et al., 2013). To raise tea-seedling rate, people often use artificial indole-3-butyric acid (IBA) and sodium naphthalene-1-acetate (NAA) to induce adventitious root formation (Samartin et al., 1986; Gunasekare and Evans, 2000; Zhou et al., 2005). However, tea-bud growth is more or less inhibited when auxins are applied. In addition, auxin residues in soil likely change soil microbial ecosystem, because auxins modulate bacterial stress resistance (Repar et al., 2013). Given that bacterial accelerator substitutes artificial phytohormones in tea-cuttage propagation, there are many advantages: (a) unlike artificial phytohormones, *Herbaspirillum sp.* WT00C can permanently promote tea-seedling growth and development, and does not inhibit tea-cutting budding; (b) it is a environment-friendly bio-accelerator, and does not cause plant disease; (d) its usage is simple and easy to handle; (e) its preparation is easy via bacterial fermentation.

IAA, produced by *Herbaspirillum* sp. WT00C, may perhaps be a major factor in stimulating lateral root formation. Ammonia and siderophore, together with IAA, may improve tea budding, tea-seedling growth and development. IAA plays an important role in cell division, elongation, coordinating cambial growth and vascular development, and initiates roots, leaves and flowers, specifically adventitious root formation in dicots or monocots (Phillips et al., 2011; McSteen, 2010). Glutamate synthase and DNA (H)-dependent glutamate dehydrogenase convert α-ketoglutarate and ammonia to glutamate in plants. This inorganic nitrogen assimilation and the subsequent steps involved in organic nitrogen supply allow the optimal growth and development of a plant (Dubis et al., 2003; Hirel, 2003; Temple et al., 1998). Siderophores, ferric ion specific chelating agents, are thought to be associated with improvement of plant growth either through a direct effect on the plant, through control of noxious organisms, or via some other routes (Neilands, 1995; Barton and Hemming, 1993). In addition, *Herbaspirillum* sp. WT00C may perhaps produce other unknown substances promoting tea-plant growth and development. There is still plainly more to be learnt about the detailed molecular mechanism of the bacterial role in microbe–host interactions. Further study on the functional genomics of *Herbaspirillum sp*. WT00C will give us a clue to fully understand its role in microbe–host interactions.

Conflict of Interests

The authors have not declared any conflict of interests.

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