APPLICATION OF BRYOPHYTE RHIZOID-ASSOCIATED BACTERIA INCREASES SILICON ACCUMULATION AND GROWTH IN MAIZE (ZEA MAYS L.) SEEDLINGS


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Abstract. Silicon (Si) enhances plant resistance to various forms of stress. However, the availability of Si as a plant nutrient is frequently limited. Bryophytes are able to grow on the surface of rocks and buildings with high Si accumulation. Therefore, bryophyte rhizoids might harbor microorganisms capable of solubilizing Si. In this study, silicate solubilizing bacteria (SSB) were isolated from the rhizoids of the bryophyte Hypnum plumaeforme L. to examine their effects on Si weathering and growth of maize (Zea mays L.). Molecular phylogeny and 16S rRNA gene sequence analysis demonstrated that the dominant bacterial strain B1-5 was a member of the Kosakonia genus. The concentrations of soluble Si released from the feldspar and quartz powder in liquid media with strain B1-5 inoculation were higher than those of the control. B1-5 inoculation in pot soil significantly increased the water-extractable Si content in soil, improved Si uptake and accumulation in maize plants, and promoted seedling growth. These results not only prove the existence of SSB in bryophyte rhizoids, but also demonstrate a new approach to searching for effective biological Si fertilizers.

Keywords: silicate solubilizing bacteria, microbial weathering, molecular phylogeny, Kosakonia, biological fertilizer

Introduction

Silicon (Si) is the second most abundant element after oxygen in soil. It occurs mostly in the form of dioxide and silicates (Myshlyaeva and Krasnoshchekov, 1974), which cannot be directly used by plants. Only water-soluble monosilicic acid [Si(OH)4], a product of the weathering of rocks and soil minerals, is available to plants (Epstein, 1994; Raven, 1983). Si has not yet been recognized as an essential nutrient element for plants, but its beneficial effects on resistance have been extensively reported in numerous crops (Epstein, 1999). In 2015 Si was listed as a “beneficial substance” by the International Plant Nutrition Institute, USA (Reynolds et al., 2016). Increasing evidences indicate that Si can alleviate both biotic and abiotic stresses including pathogens, herbivores, extreme temperature, water deficiency, high salinity and nutrient stresses (Debona et al., 2017; Ma, 2004; Wu et al., 2017). Nitrogen (N), phosphorus (P) and potassium (K) fertilizers are widely applied in agricultural production, but Si fertilizer is usually neglected. Si deficiency frequently occurs as a result of Si removal by continuous crop harvest (Liang et al., 2015). Thus, it is of significance to effectively convert unavailable Si in the soils into a form plants can utilize.
Soil microbes play a crucial role in nutrient availability and mineral weathering from primary minerals (Vessey, 2003). Most related studies focused on the P and K solubilizing abilities of some microorganisms as seen in Table 1.

**Table 1. Studies of phosphate and potassium solubilizing bacteria in crops**

<table>
<thead>
<tr>
<th>Crop name</th>
<th>Solubilizing capacity</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat (<em>Triticum aestivum</em> L.)</td>
<td>P</td>
<td>Afzal et al., 2005</td>
</tr>
<tr>
<td>Wheat (<em>Triticum aestivum</em> L.)</td>
<td>K</td>
<td>Sheng and He, 2006</td>
</tr>
<tr>
<td>Pepper (<em>Capsicum annuum</em> L.)</td>
<td>P and K</td>
<td>Han and Lee, 2006</td>
</tr>
<tr>
<td>Cucumber (<em>Cucumis sativus</em> L.)</td>
<td>P and K</td>
<td>Han and Lee, 2006</td>
</tr>
<tr>
<td>Tobacco (<em>Nicotiana tabacum</em> L.)</td>
<td>K</td>
<td>Zhang and Kong, 2014</td>
</tr>
<tr>
<td>Cotton (<em>Gossypium barbadense</em> L.)</td>
<td>K</td>
<td>Sheng, 2005</td>
</tr>
<tr>
<td>Rape (<em>Brassica campestris</em> L.)</td>
<td>K</td>
<td>Sheng, 2005</td>
</tr>
<tr>
<td>Peanut (<em>Arachis hypogaea</em> L.)</td>
<td>K</td>
<td>Youssef et al., 2010</td>
</tr>
<tr>
<td>Sesame (<em>Sesamum indicum</em> L.)</td>
<td>K</td>
<td>Youssef et al., 2010</td>
</tr>
<tr>
<td>Ryegrass (<em>Lolium rigidum</em> L.)</td>
<td>K</td>
<td>Xiao et al., 2017</td>
</tr>
<tr>
<td>Maize (<em>Zea mays</em> L.)</td>
<td>K</td>
<td>Singh et al., 2010</td>
</tr>
</tbody>
</table>

The role of silicate solubilizing bacteria (SSB), which can solubilize silicon from silicate bearing mineral and rocks (Bosecker, 1997), is largely ignored. Use of highly-efficient SSB is a promising approach for enhancing crop yield and defense in Si deficient soil.

An analysis of the Si contents in 175 plant species collected from a botanical garden shows that Bryophyta (3.46%) contains the highest Si, followed by Pteridophyta (1.4%), Gymnospermae (0.5%), Angiospermae (0.13%) (Takahashi and Miyake, 1976a, b, c), suggesting that bryophytes are Si accumulators with high Si content. Therefore, we speculated that bryophyte rhizoids harbor certain SSB capable of solubilizing Si from poor nutrient habitats.

In the present study, our aims were to: (1) isolate and characterize bryophyte rhizoids-associated silicate bacteria, (2) determine the Si-solubilizing ability of selected bacteria, and (3) examine the effect of soil inoculation with selected bacteria on Si availability in the soil, Si uptake and plant growth in maize.

**Materials and methods**

**Plants, soil and mineral**

Bryophyte *Hypnum plumaeforme* was collected from rock walls in the back hill on Jinshan Campus of Fujian Agriculture and Forestry University, Fuzhou, China (119°54′ E, 26°05′ N) in July, 2018. Bryophytes from eight random sampling locations were kept in sterilized plastic Petri-dishes until the isolation experiment began in the same day.

The soils for maize potting were collected from a hillside near the university campus. The soil was a red soil (Typic Hapludults) (Soil Survey Staff, 2010). The properties of the soil were: pH (1:2.5 w/v water) 5.5; organic C 10.03 g·kg⁻¹; total N 1.28 g·kg⁻¹; available N 19.3 mg·kg⁻¹; total P 0.13 g·kg⁻¹; available P 12.6 mg·kg⁻¹; total K 2.05 g·kg⁻¹; available K 154.6 mg·kg⁻¹, water-extractable Si 34.3 mg·kg⁻¹. Seeds of
maize (*Zea mays*, cultivar Yuecai) were obtained from the Crop Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China.

Mineral powders, feldspar (KAlSi$_3$O$_8$, SiO$_2$ 68.09%) and quartz (SiO$_2$ 98.81%), were manufactured by Foshan Hongda Ceramics Co., Ltd and passed through 0.075 mm mesh sieve. The powders were rinsed three times in pure water to eliminate soluble Si.

**Isolation of silicate bacteria from bryophyte rhizoids**

All collected bryophytes were slightly shaken to remove sands loosely adhering to the rhizoids. The rhizoids with the remaining adhered soil were swayed in 25 mL sterile water with tweezers, and the water was transferred to a 50 mL plastic tubes. The tubes were shaken with a vortex mixer for 15 s. The mixture was kept at room temperature for 30 min. The serially diluted suspension was plated on the surface of Aleksandrov’s medium for silicate bacterial culture containing 0.5% sucrose, 0.05% MgSO$_4$·7H$_2$O, 0.5% FeCl$_3$, 0.2% Na$_2$HPO$_4$, 0.01% CaCO$_3$, 0.1% KAlSi$_3$O$_8$, and 2% Agar, pH 7.2 (Hu et al., 2018). The Petri-dishes were placed in an incubator at 28 °C for 5 d. The growth of bacteria was checked every day. Fast-growing isolates with different morphology were further purified. The pure colonies were inoculated into liquid medium and preserved in 25% glycerol solution at -80 °C for future use.

**Mineral dissolution experiment**

The dominant strain was selected to test the ability to solubilize the mineral. The liquid medium consisted of 1% sucrose, 0.05% yeast extract, 0.1% NH$_4$SO$_4$, 0.2% Na$_2$HPO$_4$, 0.05% MgSO$_4$·7H$_2$O, 0.01% NaCl, 0.01% CaCO$_3$, 5 g mineral powder, pH 7.2. Each conical flask (250 mL) contained 100 mL autoclaved liquid medium and 5 mL bacterial inocula (10$^6$ cfu mL$^{-1}$) as inoculation treatment. Five mL of sterilized inoculum were also added to the liquid medium to serve as control.

Feldspar and quartz were used as Si mineral respectively in two separate experiments. The inoculation and corresponding control were replicated three times. Each flask was maintained at 180 rpm for 7 d at 28 °C. The fermented broth was centrifuged at 8000 g for 20 min. The solubilized Si concentration in the supernatant was determined using the colorimetric molybdenum blue method described by Pettersson and Karlberg (1999).

**Molecular identification**

Genomic DNA was extracted using the DNA extraction Kit (Qiagen, USA) according to the manufacturer’s protocol. The universal primers for 16S rRNA sequence amplification were 27F (5′-GTTTGATCCTGCTGCTAG-3′) and 1492R (5′-GGTACCTTGTACGACTT-3′) (Lin et al., 2012). The PCR reaction system had a volume of 25 μl, including Taq PCR Master Mix 12.5 μl, primer 2 μl, ddH$_2$O 9.5 μl, and DNA template 1 μl. The PCR was performed as follows: hot start at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. A final extension step lasted 8 min at 72 °C. The PCR products were purified and sequenced by Shanghai Biosune Biological Technology Co., Ltd. The sequencing results were analyzed using NCBI database to find the closely related homologous sequences.

The sequence and the reference sequences with the highest similarities were aligned using Clustalx 1.83 and phylogenetic analyses was done using MEGA version 4 with
the neighbor-joining method, p-distance and 1000 bootstrap replicates (Tamura et al., 2007; Thompson et al., 1997).

**Pot experiment**

Maize seeds were surface-sterilized with 10% H$_2$O$_2$ for 10 min, and then rinsed 5 times with distilled water. The seeds were soaked in distilled water for 24 h, and then pre-germinated for 48 h at 28 °C. One seedling was transplanted to a 1 L plastic pot with 750 g dry sterilized soil. The soil were autoclaved thrice in three consecutive days. Meanwhile, the cells in cultured bacterial broth were collected by centrifugation at 8000 g for 20 min at 4 °C and washed with sterilized tap water. The pelleted cells were resuspended with sterilized water and then the cells were adjusted to around $10^6$ cfu mL$^{-1}$ as determined by a standard growth curve based on optical density reading at 600 nm using a spectrophotometer (Ling et al., 2009). The experiment was established with two groups: one group was inoculated with 10 mL bacterial suspension as inoculation treatment, and the other group was added with 10 mL distilled water as control. A total of 20 pots with maize plants were divided into two groups. After 7 d, all plants were fertilized with 50 mL Hoagland nutrient solution (Hoagland and Arnon, 1950).

All plants were harvested 5 weeks after transplanting. The roots were washed and cleaned, and stem diameters were measured. All plants samples were dried at 70 °C for 3 d, then weighed. The Si contents of dry samples were then analyzed by molybdenum blue colorimetric method described by Dannon and Wydra (2004). The water-extractable Si content of air-dry soil samples (1:10 w/v water) was determined by colorimetric molybdenum blue method described by Wang et al. (2004).

**Statistical analysis**

Si concentrations in culture solution, growth indexes and Si content of plants and soils were analyzed using t-test (P < 0.05) to determine difference between bacterial inoculation and control. All analyses were performed with the SPSS 13.0 software package (SPSS Inc.).

**Results and discussion**

**Isolation silicate bacteria from the bryophyte rhizoids**

Si-solubilizing bacterial isolates were obtained from the bryophyte Hypnum plumaeforme rhizoids on rock walls (Fig. 1A), and one of them showed the dominant colony which was named as B1-5, and kept for further study. On the Aleksandrov plates, the strain B1-5 formed round, smooth, convex, slimy, elastic, translucent and colorless colonies (Fig. 1B). Silicate solubilizing bacteria (SSB) can solubilize silicon from silicate mineral (Bosecker, 1997). Bryophyte with high Si-accumulation mostly can grow on the surface of rocks and buildings. So it raises the possibility that some SSB may adhere to the bryophyte rhizoids.

**Molecular identification based on 16S rRNA analysis**

About 1500 bp band was obtained from the amplification of 16S rRNA gene sequence from the strain B1-5. The 16S rRNA gene sequence of B1-5 clones was submitted to GenBank with accession number MH051262. Phylogenetic analysis
showed that B1-5 and all 6 Kosakonia species formed a branch, and that other outgroup species formed different branches (Fig. 2). Thus, the B1-5 strain belongs to the Kosakonia genus. Figure 2 also showed that strain B1-5 shared the highest sequence similarities with Kosakonia cowanii. Thus, strain B1-5 was identified as a member of Kosakonia.

Several Kosakonia species isolated from soils and trees promote plant growth (Brady et al., 2013). Diazotrophic bacteria, K. pneumoniae and K. radicincitans were found to be associated sugarcane at Northeast Region of Brazil (Antonio et al., 2016). K. radicincitans improves fruit yield and quality of Solanum lycopersicum (Berger et al., 2017). K. radicincitans alters Arabidopsis thaliana root and root exudate metabolism (Witzel et al., 2017). K. pseudosacchari sp. nov., an endophyte of Zea mays was isolated (Kaempfer et al., 2016).

**Si solubilizing activity of the bacteria**

The ability of strain B1-5 to solubilize Si was assessed in liquid media containing mineral powders of KAlSi₃O₈ and SiO₂ (Fig. 3). After 7 days of incubation, a significant increase in soluble Si concentration of the medium with KAlSi₃O₈ (t = -5.344, df = 4, P = 0.006) or SiO₂ (t = -43.466, df = 4, P < 0.001) supplement was observed in inoculated versus control seedlings.
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Figure 2. Phylogenetic dendrogram based on comparative analysis of the 16S rRNA gene sequence, showing the relationship among strain B1-5 and other related species and genera. The GenBank sequence accession numbers were indicated in brackets after the strain names. The dendrogram was generated using the neighbour-joining method in the bootstrap test (1000 replicates).

Figure 3. Si content in KAlSi₃O₈ - (A) and SiO₂-containing (B) liquid medium with and without inoculation with the isolated silicate bacterium strain B1-5. Si content was determined 7 d after inoculation with strain B1-5. Significant differences (P < 0.05) between inoculation and non-inoculated control are indicated by asterisk above bars. Values are mean ± standard error from three replicates.
Here we found the B1-5 bacterial strain was able to solubilize silicate from Si-bearing mineral in fermentation liquid. Indeed, microbial leaching is a simple and effective technology for metal extraction from low-grade ores and mineral concentrates, and this method is being increasingly applied in mining industry (Bosecker, 1997). Mineral weathering mechanism has been increasingly clarified in terms of chemical weathering processes. These microbes have been indicated to accelerate the dissolution of silicates by perturbing mineral–water equilibria and reaction dynamics, the production of chelating agents, proton, organic ligands, hydroxyl anion, extracellular polysaccharides and enzymes, and silicate precipitation by metal sorption at the cell membrane (Bennett et al., 2001). However, the influence of bacteria in this process and its molecular mechanisms are still unclear (Uroz et al., 2009).

**Effect of strain B1-5 on maize growth and Si content in soil and plants**

Maize seedlings grown in soils inoculated with B1-5 showed greater shoot diameter ($t = -4.745$, $df = 18$, $P < 0.001$) and dry biomass ($t = -2.634$, $df = 18$, $P = 0.017$) than uninoculated plants (Fig. 4). Soil inoculation with B1-5 strain significantly increased the growth of maize plants.

![Figure 4: Shoot diameter (A) and biomass (B) of maize seedlings grown in the soil with and without inoculation of strain B1-5. Significant differences ($P < 0.05$) between inoculation and non-inoculated control are indicated by asterisk above bars. Values are mean ± standard error from ten replicates](image)

The soluble Si content in the soil inoculated with B1-5 strain was higher than that in uninoculated control ($t = -3.67$, $df = 18$, $P = 0.001$) (Fig. 5A). Maize plants grown in inoculated soil showed significantly higher Si level in shoots ($t = -2.55$, $df = 18$, $P = 0.020$) and roots ($t = -2.228$, $df = 18$, $P = 0.039$) than those in uninoculated plants (Fig. 5B). Inoculation with B1-5 strain significantly increased soluble Si release from the soil and Si uptake by maize plants.

Our study clearly illustrated that the growth and Si content of maize plants were improved by inoculation with B1-5 strain via enhancement of soil Si availability and plant Si uptake. This is consistent with the findings that SSB, *Bacillus circulans* *Burkholderia eburnean* and *Enterobacter ludwigi*, increased the release of Si in soil and Si acquisition by plants (Kang et al., 2017; Lee et al., 2019; Zahra et al., 1984). Many studies have
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showed that Si application improves the growth of monocot crops including rice, sugarcane (Saccharum officinarum) and turf (Lolium perenne) (Nanayakkara et al., 2008; Savant et al., 1999; Snyder, 2001). Moreover, two field experiments in India have also reported that silicon solubilizing bacteria improve silicon uptake and crop yield in rice and sugarcane (Brindavathy et al., 2012; Pedda et al., 2016).

**Figure 5.** (A) Soluble Si content in strain B1-5-inoculated and un-inoculated soils. (B) Si content in the shoots (left) and roots (right) of maize seedlings grown in strain B1-5-inoculated and un-inoculated soils. Significant differences (P < 0.05) between inoculation and control are indicated by asterisk by different letters above bars. Values are mean ± standard error from ten replicates.

**Conclusion**

In conclusion, this study not only highlights the existence of bryophyte rhizoids-associated silicate solubilizing bacteria, but also opens up a new approach searching for Si-solubilizing bacteria to increase Si availability in soil. Use of silicate solubilizing bacteria biofertilizer may serve as an important approach to increase Si availability and crop production. More detailed mechanisms of microbiological molecular biology and metabonomics about mineral weathering await further research.

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