

Bacillus Benefits the Growth of Ambrosia Artemisiifolia by Increasing Available Nutrient Levels

Fengjuan Zhang (✉ fengjuanzhang@126.com)

Hebei University <https://orcid.org/0000-0002-7131-2592>

Jianru Sun

Hebei University

Chang Wang

Hebei University

Chunying Li

Hebei University

Xue Chen

Hebei University

Haiyun Xu

Hebei University

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Abstract

Aims

Bacillus, a gram-positive bacterium, has multiple beneficial traits which help the plants in nutrients acquisition, either directly or indirectly. However, the mechanisms that mediate the positive or negative impact of *Bacillus* on exotic or native species are poorly understood. Our objective was to determine whether the quantitative and/ or qualitative differences in the *Bacillus* community present on the exotic *Ambrosia artemisiifolia* and the native *Setaria viridis* provide a competitive advantage to the invader over native species.

Methods

A. artemisiifolia monoculture, mixture of *A. artemisiifolia* and *S. viridis* and *S. viridis* monoculture were designed in the field experiment. *Bacillus* diversity in their rhizospheres was analyzed using 16S rRNA and their effects on the competitive growth of *A. artemisiifolia* and *S. viridis* were tested in greenhouse experiment.

Results

The Shannon index, species richness, and evenness index of *Bacillus* diversity in the rhizosphere soil of *A. artemisiifolia* in the monoculture treatment were lower than in the mixture treatment. The relative abundance of *Bacillus megaterium* in the rhizosphere soil of *A. artemisiifolia* was higher than that in the rhizosphere soil of *S. viridis*. Whether *Bacillus* in the rhizosphere soil of *A. artemisiifolia* or *B. megaterium* inoculation enhanced the relative competitiveness of *A. artemisiifolia* and inhibited that of *S. viridis* by altering their carbon, nitrogen, and phosphorus concentrations.

Conclusions

A. artemisiifolia invasion influenced *Bacillus* communities, especially *B. megaterium*. The higher abundance of *B. megaterium* in *A. artemisiifolia* rhizosphere creates higher levels of the available nutrient than that in native *S. viridis*.

Introduction

Invasive plant species often outcompete native species when colonizing new ranges. In principle, the growth and expansion of a plant species in a diverse community of competing organisms are dependent on two components: its ability to absorb nutrients from the soil and its impact on the available soil resources (Chase and Leibold 2003). According to some studies, invasive plant species have a higher nutrient acquisition capacity than native species. This difference has been attributed to the fact that the photosynthetic nitrogen use efficiency and photosynthetic energy use efficiency of invasive species are higher than that of native species in their invaded range (Pattison et al. 1998; Baruch and Goldstein 1999; Feng et al. 2008). Some studies also pointed out that invasive species adapt faster to different levels of

nutrient environments and have a higher ability to modify resources than the native species, thus their successful invasion (Davidson et al. 2011; Parker et al. 2013; Luo et al. 2019). Upon the successful invasion of a new range, the invasive plant species may exploit unused resources (Shea and Chesson 2002) or modify resources to benefit themselves, or weaken the growth of other species (Chase and Leibold 2003; Parker et al. 2019). Therefore, it is crucial to investigate the differences in invasive and native plant species potential to modify resources during an invasion.

Soil microbial communities are essential in the soil nutrient cycle (Fraterrigo et al. 2006; Iwaoka et al. 2018). The rhizosphere microbiome is involved in important processes, such as nitrogen fixation, mobilization of phosphorus, and alteration of other nutrients (Rodríguez-Caballero et al. 2020). Moreover, invasive plants can alter the microbial community in the rhizosphere and accumulate some beneficial microorganisms in their rhizospheres, such as *Bacillus*, arbuscular mycorrhizal fungi and *Pseudomonas* (Ehrenfeld 2003; Kourtev et al. 2003; Gibbons et al. 2017). *Bacillus* is one of the rhizosphere-promoting bacteria genera known as a natural plant nutrition resource (Saxena et al. 2019). Besides, members of the genus *Bacillus* are known to have multiple beneficial traits which directly or indirectly aids plants to acquire nutrients (Saxena et al. 2019). Different *Bacillus spp.* had different ability to fix nitrogen, solubilize and mineralize phosphorus (Goswami et al. 2016; Pramanik et al. 2019). Moreover, root exudates are among the important factors that influence those abilities (Tariq et al. 2007; Zhang et al. 2014; Shakeel et al. 2015; Wang et al. 2020). The difference between the root exudates of invasive and native species may enable the invasive species to recruit specific *Bacillus* species in the rhizosphere of the invasive plant species. The bacteria help the invading species exploit more available resources in the soil and eventually become the dominant plant species. However, only a few studies have investigated the interaction between exotic species and *Bacillus* species (Dai et al. 2016; Sun et al. 2020). Therefore, understanding the differences in *Bacillus* communities and function between native and exotic populations would help predict the ecological roles of *Bacillus* communities in plant invasion.

The exotic species *Ambrosia artemisiifolia*, commonly known as ragweed, belongs to the family Asteraceae and is widely distributed in China (Xu and Qiang 2004). Its invasion poses a serious threat to biodiversity and agricultural production (Ozaslan et al. 2016). In addition, it often causes human health problems due to its allergenic pollen (Ghiani et al. 2012). According to Zhang et al. (2018), *A. artemisiifolia* invasion changes the rhizosphere microbial community in its rhizosphere soil. Specifically, when *A. artemisiifolia* grows with native *Setaria viridis* (L.) Beauv. (Poaceae) the increased AMF colonization in *A. artemisiifolia* and the decreased AMF colonization in *S. viridis* provide a competitive advantage to the invasive species over the native species (Zhang et al. 2018). Previous studies have shown that *Bacillus spp.* is often found in the rhizosphere soil of invasive plants (Huangfu et al. 2015; Song et al. 2017). To test the role of *Bacillus spp.* in the invasion of *A. artemisiifolia* we hypothesized that: (i) the *Bacillus* community in the *A. artemisiifolia* rhizosphere differs from that in the rhizosphere of co-occurring native species; and (ii) the assembly of specific *Bacillus* species benefits the invasive species than the native species. Then, we conducted three sets of experiments to test our hypotheses. First, *Bacillus* was separately isolated from the rhizosphere soil of *A. artemisiifolia* and *S. viridis* to compare their effect on the competitive growth of *A. artemisiifolia* in a greenhouse experiment. Then a comparison

of *Bacillus* diversity was performed to determine whether *A. artemisiifolia* recruits specific *Bacillus* species in its rhizosphere soil. Lastly, the role of specific *Bacillus* species in the competitive growth of *A. artemisiifolia* was evaluated in a greenhouse experiment. The present study results provide a broader understanding of the functional role of *Bacillus* in promoting the invasion of *A. artemisiifolia*.

Materials And Methods

Experiment I: Comparative analysis of the effect of *Bacillus* from the rhizosphere soil of *A. artemisiifolia* and *S. viridis* on the competitive growth of *A. artemisiifolia*

Experimental design

A long-term field experiment was established at the Langfang Experimental Station, Chinese Academy of Agricultural Science (CAAS), Beijing, China (39° 30′ 42″ N, 116° 36′ 07″ E), about 10 km southeast of Beijing. Our experimental design was as described by Zhang et al. (2018). *S. viridis* is an annual C₄ monocotyledonous species that is widely distributed in areas invaded by *A. artemisiifolia*. The experimental plots (3 m × 2 m) were prepared in 2008, with a 1 m isolation zone to prevent edge effects. Three treatments used in the experiment included: (1) *S. viridis* monoculture (S), (2) an equal mixture of *A. artemisiifolia* and *S. viridis* (1:1; A:S), and (3) *A. artemisiifolia* monoculture (A). Each treatment had five replicates. Without tilling, the plots were hand weeded to leave bare soil before sowing. The soil was watered to maintain a soil field capacity of 40%. The seeds were sterilized in 10% H₂O₂ for 2 min, rinsed with sterile dH₂O for 2 min, and then rinsed with dH₂O five times for 5min. In May 2008, 100 seeds were sown in each plot to begin the experiment (for the mixture (A:S), we used 50 *A. artemisiifolia* seeds and 50 *S. viridis* seeds). No fertilizers were applied, and the plant species composition in each plot was maintained by manually removing the weeds throughout the experiment period annually. Each spring of every year, all plants were pulled out of the soil, and the plots were reseeded to ensure that the target plants (*A. artemisiifolia* and *S. viridis*) would be exposed to the desired treatment conditions every year.

Soil sampling

After eleven years, the abundance of each plant species had changed, so we reduced the number of replicates to three, with similar plant aerial cover. In 2019, the cover of *A. artemisiifolia* and *S. viridis* in the monocultures was 99% and 93%, respectively, and 76% and 20%, respectively, in the mixed treatment. We followed Huber (2011) to identify the outliers of plants and thinned samples to obtain a sample that would robustly reflect the mean. We visually estimated the mean and chose plants that seemed to be typical in each treatment. In each treatment, five plants were selected per species and pooled together for subsequent index determination. We brushed off the closely adhered soil, which serves as rhizosphere soil, after shaking off the loosely bound soil. The rhizosphere soil from the three treatments was pooled per plot per plant species. The control soil was collected after the removal of the surface soil using five-point sampling. Three plots were sampled for each of the five treatments, yielding a total of 15 soil samples (3 treatments × 5 replicates = 15). The soil samples were collected and sieved (< 0.4 mm). For

the *Bacillus* diversity analysis, 6 g of the soil samples was stored at -80°C, while 10 g was stored at 4°C in sterile containers for the *Bacillus* inoculum experiment.

Bacillus isolation from the rhizosphere soil

Bacillus was isolated from the rhizosphere soil of *A. artemisiifolia* and *S. viridis*, separately. Briefly, 1 g of sample soil was added to 9 mL sterile distilled water in a sterilized conical flask. The suspension was homogenized and heated in a hot water bath at 90°C for 10 min. After 12 h, 4 mL of supernatant was aspirated and filtered through a membrane filter with a diameter of 0.22 µm to remove allelochemicals and other organic compounds. The *Bacillus* remained on the membrane and was rinsed five times with 5 mL of sterile distilled water. The *Bacillus* suspension was cultured on the beef extract peptone liquid medium while shaking (180 rpm) for 24 h at 37°C. The optical density of the suspension was adjusted to approximately 1.0 (O.D at 600 nm) by diluting it with sterile distilled water. The population count of *Bacillus* was maintained at 10⁸cfu/mL.

Bacillus inoculation design

Three treatments were used to determine the effect of the *Bacillus* isolated from the rhizosphere of *A. artemisiifolia* and *S. viridis* on the competitive advantage they provide to the former. (1) Monoculture of *A. artemisiifolia* (A), (2) monoculture of *S. viridis* (S), and (3) an equal mixture of *A. artemisiifolia* and *S. viridis* (A:S). Each treatment was further divided into three levels: (1) C, uninoculated treatment; (2) AA, inoculated with *Bacillus* isolated from the rhizosphere of *A. artemisiifolia*; and (3) SV, inoculated with *Bacillus* isolated from the rhizosphere of *S. viridis*. Seeds of *A. artemisiifolia* and *S. viridis* were obtained from the Langfang Experimental Station, surface sterilized with 75% ethanol, and washed with sterile distilled water five times before sowing in pots (10 × 10 × 12 cm). The soil in the box was composed of sandy clay and vermiculite (v/v, 1:1). The sandy clay was collected near the experimental field site from an open area that had not been covered with vegetation for the previous three years. Vermiculite was obtained from Baisheng Plant and Flower Co., Ltd., Baoding, China. Basic soil properties included: pH (w/v water = 1:5) of 8.20, organic matter content of 14.29 g/kg, available nitrogen of 52.18 mg/kg, and available phosphorus of 3.4 mg/kg.

Seeds were planted in pots with 1 kg soil, and the respective bacterial suspensions (10 mL 10⁸cfu/mL) were added to initiate the experiment. The bacterial suspension was added once a month. After germination, each pot was weeded to contain only four plants (four per species in the monocultures and two per species in the mixed treatment in a substitution design). Each treatment was replicated ten times (3 × 4 treatments × 10 replicates = 120 pots). The pots were placed in a greenhouse for 90 days under a 10-h light: 14-h dark photoperiod at 25°C, arranged in a completely randomized design.

Measurements

The plants grown under the different treatments were harvested after 90 days. The roots were washed free of soil and then oven-dried at 80°C for 48 h to collect growth index data. The entire plant, including

aboveground biomass and root biomass, was used to determine dry biomass. The corrected index of relative competition intensity (CRCI) was calculated as described by Oksanen et al. (2006).

CRCI = arcsine [(X-Y)/max (X, Y)], where X is the average biomass of individual plants grown without competition and Y is the biomass which grown in competition.

Experiment II: Comparative analysis of the *Bacillus* diversity in the rhizosphere soil of *A. artemisiifolia* and *S. viridis*

Bacillus isolation

We isolated *Bacilli* from the soil samples as described by Chikerema et al. (2012), with modification. Briefly, soil slurry was prepared by suspending 1 g of soil in 9 mL sterile water. The suspension was homogenized and cultured for 24 h at 25°C and 180 rpm in an incubator (BSD-100, Shanghai Boxun Industry & Commerce Co., Ltd). The soil suspension was serially diluted after being heated in a water bath at 90°C for 10 min. Appropriate dilutions were cultured on beef paste with a peptone medium (10 g peptone, 3 g beef paste, 5 g sodium chloride, and 1000 mL distilled water. pH: 7.2–7.4, 121°C for 30 min) using the spread-plate method. The plates were cultivated at 30°C for 36 h. Based on the number of *Bacilli* and their separation, 10^{-3} g/mL of the suspension was used to isolate the bacteria. All the colonies in the plates were picked and purified on nutrient-agar plates.

Analysis of *Bacillus* diversity

DNA extraction A single colony was picked from the plate and transferred into a 1.5 mL microfuge tube containing a liquid medium (10 g peptone, 3 g beef paste, 5 g sodium chloride, and 1000 mL distilled water. pH: 7.2–7.4, 121°C for 30 min). The homogenized bacterial culture was precipitated by centrifugation at $28600 \times g$ for 10 min at 4°C. The supernatant was discarded, and 400 μ L 10 \times TE (1 M Tris-HCl: 0.5 M EDTA = 5:1) was added to the tube. The cells were homogenized twice in a bead-beater (Mini-Bead Beater 8™, Biospec Products, Bartlesville, OK, USA) at 4.5 speeds (200 rpm) for 45 s then centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was discarded, and 400 μ L 10 \times TE was added to eliminate the protein. After centrifugation, the supernatant was removed, and 400 μ L 10 \times TE and 25 μ L lysozyme (50 mg/mL) was added to the tube. The cells were then incubated for 12 h at 37°C. Thereafter, 100 μ L 10 % SDS was added, and the cells were homogenized twice in a bead-beater at 4.5 speeds (200 rpm) for 45 s. An aliquot (400 μ L) of phenol-chloroform-isoamyl alcohol 25:24:1 (Sigma, Milan, Italy) was added and centrifuged at $28600 \times g$ for 10 min at 4°C. The supernatant was transferred to a 1.5-mL tube, and 400 μ L ice-cold (- 20°C) chloroform-isoamyl (24:1) was added, followed by centrifugation at $28600 \times g$ for 10 min at 4°C. The supernatant was transferred into a 1.5-mL tube, and 0.5 mL ice-cold absolute ethanol (100%) was added to the tube. After incubation for 1 h at - 20°C, DNA was precipitated by centrifugation at $28600 \times g$ rpm for 10 min at 4°C. The pellet was then washed with 200 μ L 70 % ethanol and centrifuged. The supernatant was removed, and the pellet was then air-dried. The NanoDrop 2000 ultra-microspectrophotometer was used to determine the quality and concentration of DNA.

16S rRNA gene amplification and sequencing The 16S rRNA gene was commercially sequenced to identify the isolated *Bacilli*. The full-length gene was amplified from the bacterial DNA using universal primers F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and R1492 (5'-GGTTACCTTGTTACGACTT-3'). A PCR mixture of 50 µL volume was conducted, comprising 2 µL *Bacillus* DNA, 0.6 µL deoxynucleotide triphosphate, 3 µL PCR buffer, 0.3 µL each of reverse and forward primer, 0.3 µL Es Taq DNA polymerase, and 23.5 µL distilled water. The PCR reaction was carried out in a T100 thermocycler (BIO-RAD, USA). PCR conditions included: 30 cycles at 94°C for 1 min, 60°C for 45 s, and 72°C for 1 min, followed by a final extension at 72°C for 8 min. The amplified PCR product (1500 bp) was separated via gel electrophoresis on 1% (w/v) agarose gel. The sequencing was performed commercially by the General Biosystems (Anhui, China).

Phylogenetic analyses The obtained 16S rDNA sequences were assembled using DNASTar (Lasergene, Madison, WI, USA). Sequences were imported into EZBioCloud (<http://www.ezbiocloud.net>) and subjected to a BLAST homology search with reference to a known 16S rDNA sequence. Species-level identification was performed based on a 16S rDNA sequence similarity of $\geq 97\%$ to that of a prototype strain sequence in the GenBank. Clustal W (2.0.10) in BioEdit was used to perform multiple sequence alignment for the sequence and several near-margin sequences. Sequences were aligned with other published *Bacilli* sequences, and neighbor-joining phylogenetic analyses were done using MEGA v5.05 (Tamura et al., 2011). Distances in the neighbor-joining tree were computed using the default parameters. Phylogenetic trees were generated under maximum parsimony, neighbor-joining with Jukes-Cantor correction, and a maximum likelihood algorithm with 1000 bootstrap replicates. The sequences reported in the experiment were deposited in the EzTaxon database (<https://www.ezbiocloud.net/>) under the accession numbers MW759418–MW759434.

Bacillus community diversity The relative abundance (*RA*) of *Bacilli* in our entire sample (including non-*Bacillus* DNA amplified by the *Bacillus* primers) was calculated as:

$RA = A/N \times 100\%$, where *A* indicates the number of sequences of one *Bacillus* phylotype, and *N* indicates the total number of sequences.

The Shannon index (*H*), species richness (*D*), and evenness index (*E*) were calculated as additional measures of *Bacillus* diversity. The formulae included:

$$H' = -\sum_{i=1}^S p_i \ln p_i$$

$$p_i = n_i / N,$$

where *S* represents the total number of *Bacillus* phylotypes, *n_i* represents the number of *Bacillus* phylotype *i*, and *N* represents the number of all *Bacillus* phylotypes.

$$D = (S - 1) / \ln N$$

$$E = H / \ln S$$

Experiment III: Effect of specific *Bacillus* species (*Bacillus megaterium*) on the competitive growth of *A. artemisiifolia*

B. megaterium inoculation

B. megaterium strains were isolated from the rhizospheres of *A. artemisiifolia*. The previously refrigerated (-20°C) *Bacillus* was thawed in a water bath at 30°C for 90 s. An aliquot of the bacterial suspension was spread in a petri dish of beef paste with a peptone medium. The petri dish was cultivated at 37°C for 12–24 h. Subsequently, a single colony was inoculated into 1 mL liquid medium at 30°C for 24 h. Then, 1 mL *B. megaterium* suspension was inoculated into 100 mL liquid medium at 30°C on a rotary shaker for 24 h at 180 r/min to attain a spore concentration of 1×10^8 colony-forming units (CFU) mL⁻¹.

Experimental design

Three treatments, including *A. artemisiifolia* monoculture (Am), *S. viridis* monoculture (Sm), and an equal mixture of *A. artemisiifolia* and *S. viridis* (A:S), were used to determine the effects of *B. megaterium* on the competitiveness of *A. artemisiifolia* over *S. viridis*. The seeds were added to pots with 1 kg of soil. After seed germination, each pot was thinned out to four plants (4 individuals in the monocultures and two species each in the mixed treatments in a substitution design). Four densities of *B. megaterium* (C0: 0, C1: 5×10^8 cfu/mL, C2: 15×10^8 cfu/mL, C3: 30×10^8 cfu/mL of *B. megaterium*) were tested. The bacterial suspensions were added monthly, as described by Du et al. (2020). Meanwhile, the uninoculated treatment (C0) was treated with 1 mL sterile water and 100 mL liquid medium. Each treatment had 10 replicates (3 × 3 treatments × 10 replicates = 90 pots). The pots were placed in a greenhouse for 90 days under a 10 h photoperiod at 25°C in a randomized design. No fertilizer was used, and each pot was regularly weighed to maintain the soil water content at 25%.

Plant growth parameters

A. artemisiifolia and *S. viridis* grown in different treatments were harvested 90 days after germination. The soil was washed from the roots, and the plants were oven-dried at 80°C for 48 h to collect growth index data. The whole plant, including aboveground and root biomass, was used to determine dry biomass. Total dry plant weight data of *A. artemisiifolia* and *S. viridis* were recorded. The corrected index of relative competition intensity (CRCI) was used to quantify the effect. Samples weighing 20 mg each of *A. artemisiifolia* and *S. viridis* leaves and stems were analyzed to determine the total carbon content. The carbon content was measured using the potassium dichromate-concentrated sulfuric acid (K₂Cr₂O₇-H₂SO₄) oxidation method. Dry *A. artemisiifolia* and *S. viridis* matter (2 g each) were digested in a mixture of concentrated perchloric and nitric acids (v:v = 1:6) to analyze total nitrogen and phosphorus contents. The nitrogen content was determined using the micro-Kjeldahl method (Nelson and Sommers 1972), while the phosphorus content was measured using inductively coupled plasma spectroscopy (Isaac and Johnson 1983). Each experiment was replicated ten times.

Available nutrients

Available phosphorus (AP) extracted by 0.5 M NaHCO₃ was measured by the molybdenum blue method, while available potassium (AK) extracted by 1 M CH₃COONH₄ was measured using the flame photometry method (Shi et al. 2018).

B. megaterium density

The density of *B. megaterium* was determined to compare the growth of *B. megaterium* among different treatments. This was done for each fresh soil sample using serial dilution techniques on agar plates with nutrient broth medium. Specifically, when plants were harvested, a 1 g soil sample was collected from the rhizosphere soil and transferred to a tube, and 9 mL distilled water was added. The suspension was shaken to homogeneity at 200 r/min for 24 h, then heated in a hot water bath at 90°C for 10 minutes. After 12 h standing, the supernatant was serially diluted from 10⁻² to 10⁻⁵, then up to 0.1 mL was pipetted from each aseptic dilution using a flattened micropipette and added to nutrient agar plates. The plates were incubated at 37°C for 12 h. The density of *B. megaterium* was estimated by counting the single colonies. The *Bacillus* concentration was determined based on the number of colonies and colony separation in the 10⁻³ dilution. Then the cfu/1 g dry weight of soil (cfu/g DWs) was calculated according to the volume dilution. Each treatment was replicated thrice.

Statistical analysis

One-way ANOVA was performed to test the differences in *Bacillus* diversity between the *A. artemisiifolia* and *S. viridis* rhizosphere soils in different treatments, as well as the effect of *Bacilli* isolated from the rhizosphere soil of *A. artemisiifolia* or *S. viridis* on the CRCI of each species. Two-way ANOVA was used to analyze the effect of competition and *Bacillus* on plant growth parameters, concentrations of available nitrogen and phosphorus in the soil, and the density of *Bacillus*. All the analyses were performed using IBM SPSS Statistics 19.0 (IBM Corp., Armonk, NY, USA). The Tukey Honest procedure was applied to compare means between treatments.

Results

Comparative analysis of the effect of *Bacillus* from the rhizosphere soil of *A. artemisiifolia* and *S. viridis* on the competitive growth of *A. artemisiifolia*

Compared to the monoculture of *A. artemisiifolia*, the total dry biomass of *A. artemisiifolia* in the mixture treatment increased ($F = 108.495$, $P < 0.001$) (Fig. 1). When *Bacillus* from the rhizosphere soil was inoculated, the biomass of *A. artemisiifolia* was significantly higher than that of the uninoculated treatment ($F = 99.723$, $P < 0.001$). Furthermore, the biomass of *A. artemisiifolia* inoculated with *Bacillus* from *A. artemisiifolia* soil was higher than that from *S. viridis* soil ($P < 0.001$). However, the biomass of *S. viridis* inoculated with *Bacillus* from *A. artemisiifolia* soil was lower than that from *S. viridis* soil ($P <$

0.001). CRCI of *A. artemisiifolia* further showed that *Bacillus* in the rhizosphere soil of *A. artemisiifolia* enhanced its competitive growth (Fig. 2).

Bacillus diversity in rhizosphere soil

The *Bacillus* diversity varied among the different treatments. For instance, seven and eleven *Bacillus* phylotypes were identified in the rhizosphere soil of *A. artemisiifolia* in A and A:S. Meanwhile, the rhizosphere soil of *S. viridis* in S and A:S had ten and six *Bacillus* phylotypes, respectively (Table S1). The Shannon index, species richness, and evenness index of *Bacillus* diversity in the rhizosphere soil of *A. artemisiifolia* in A treatment was lower than the A:S treatment (all, $p < 0.05$). However, *Bacillus* in the rhizosphere soil of *S. viridis* in S treatment was higher than that in A:S treatment (all, $p < 0.05$) (Table 1). *Bacillus megaterium*, *Bacillus aryabhattai*, and *Bacillus bingmayongensis* were the dominant species in all the soil samples, with *B. megaterium* having the highest relative abundance in the rhizosphere soil of *A. artemisiifolia* in A (Fig. 3).

Effect of *B. megaterium* on the competitive growth of *A. artemisiifolia*

Biomass and CRCI

There was no significant change in the biomass of *A. artemisiifolia* between the monoculture and mixed treatment (with *S. viridis*) in the uninoculated (control) treatments ($P = 0.228$). Conversely, the competition with *A. artemisiifolia* decreased the biomass of *S. viridis* ($F = 136.15$; $P < 0.001$) (Fig. 4, Table S2). In both plant species, the biomass increased with the increase of the density of *B. megaterium* ($P < 0.05$), showing a dose-dependent relationship. Our analysis showed that the biomasses of C2 and C3 inoculation treatments were significantly higher than the uninoculated treatment (all, $P < 0.001$). Compared to the monoculture, competition had different effects on the biomass of *A. artemisiifolia* and *S. viridis* in the inoculation with C2 and C3 *B. megaterium*; the biomass of *A. artemisiifolia* increased ($F = 65.091$; $P < 0.001$), while that of *S. viridis* decreased ($F = 136.15$; $P < 0.001$). CRCI results further showed inoculation with C2 and C3 *B. megaterium* concentrations enhanced the relative competitiveness of *A. artemisiifolia* ($F = 12.996$; $P < 0.001$) while decreasing that of *S. viridis* ($F = 7.569$; $P = 0.002$) (Fig. 5).

Total carbon, nitrogen, and phosphorus concentrations of *A. artemisiifolia* and *S. viridis*

B. megaterium had a dose-dependent effect on total carbon, nitrogen, and phosphorus concentrations of *A. artemisiifolia* and *S. viridis* (Fig. 6, Table S2). There was no significant difference between C0 and C1 treatments (Carbon: A: $P = 0.081$; S: $P = 0.108$; Nitrogen: A: $P = 0.537$; S: $P = 0.1$; Phosphorus: A: $P = 0.183$; S: $P = 0.331$). However, in the monoculture of both C2 and C3 treatments, the total carbon, nitrogen, and phosphorus concentrations of *A. artemisiifolia* or *S. viridis* were significantly higher than in C0 treatment (C2: Carbon: A: $P < 0.001$; S: $P < 0.001$; Nitrogen: A: $P < 0.001$; S: $P < 0.001$; Phosphorus: A: $P < 0.001$; S: $P < 0.001$. C3: Carbon: A: $P < 0.001$; S: $P < 0.001$; Nitrogen: A: $P < 0.001$; S: $P < 0.001$; Phosphorus: A: $P < 0.001$; S: $P < 0.001$). Compared to the monoculture, the total carbon, nitrogen, and phosphorus concentrations of

A. artemisiifolia in the mixture increased (Carbon: A: $F = 55.737$; $P < 0.001$; Nitrogen: A: $F = 92.315$; $P < 0.001$; Phosphorus: A: $F = 23.285$; $P < 0.001$). However, the concentrations in *S. viridis* decreased in both uninoculated and inoculated treatments (Carbon: S: $F = 46.598$; $P < 0.001$; Nitrogen: S: $F = 96.474$; $P < 0.001$; Phosphorus: S: $F = 58.443$; $P < 0.001$).

Available nitrogen and phosphorus concentrations in the soil of different treatments

No significant difference in available nitrogen and phosphorus concentration in the soil was revealed between the C0 and C1 treatments (Phosphorus: A: $P = 0.181$; S: $P = 0.628$; A:S: $P = 0.454$; Nitrogen: A: $P = 0.337$; S: $P = 0.292$; A:S: $P = 0.153$) (Table S2). However, in both C2 and C3 treatments, the total carbon, nitrogen and phosphorus concentrations of *A. artemisiifolia* or *S. viridis* were significantly higher than in C0 treatment (C2: Phosphorus: A: $P < 0.001$; S: $P < 0.001$; A:S: $P < 0.001$; Nitrogen: A: $P < 0.001$; S: $P < 0.001$; A:S: $P < 0.001$; C3: Phosphorus: A: $P < 0.001$; S: $P < 0.001$; Nitrogen: A: $P < 0.001$; S: $P < 0.001$). The total carbon, nitrogen, and phosphorus concentrations of *A. artemisiifolia* in the mixture increased compared to the monoculture, while *S. viridis* concentrations decreased in both uninoculated and inoculated treatments (Phosphorus: S: $F = 59.462$; $P < 0.001$; Nitrogen: S: $F = 9.865$; $P < 0.001$).

The density of *B. megaterium* in different treatments

A dose-dependent increase in soil *B. megaterium* levels was observed when grown in monoculture (A or S) or mixture (A: S). For the *A. artemisiifolia* monoculture, the density of *B. megaterium* in C2 and C3 treatments was significantly higher than the C0 treatment ($F = 35.944$, $P < 0.001$). Meanwhile, the density of *B. megaterium* in C3 treatments of *S. viridis* monoculture was significantly higher than all the others ($P < 0.001$). It was also found that the density of soil *B. megaterium* in the mixture treatment was significantly higher than in the *A. artemisiifolia* ($F = 49.849$, $P < 0.001$) and *S. viridis* ($F = 47.811$, $P < 0.001$) monocultures.

Correlation of the density of *B. megaterium* with plant growth indicators

The density of *B. megaterium* was positively correlated with the growth indicators of *A. artemisiifolia* in all the treatments (Table S3). In *S. viridis*, the density of *B. megaterium* was positively correlated with the biomass, total carbon, nitrogen, and phosphorus content in the monoculture but was not correlated with the growth indicators of *S. viridis* in the mixture.

Discussion

We aimed to determine whether *Bacillus* in the rhizosphere soil of *A. artemisiifolia* enhances the competitive growth of the exotic species. Rhizosphere soils were inoculated with *Bacillus* to provide an advantage to the invasive species over the native *S. viridis*. Our study shows that the effects of *Bacillus* in the rhizosphere soil of *A. artemisiifolia* and in the rhizosphere soil of *S. viridis* on plant growth are different. For instance, *Bacillus* in the rhizosphere soil of *A. artemisiifolia* enhanced the competitive

growth of *A. artemisiifolia*. In contrast, *Bacillus* in the rhizosphere soil of *S. viridis* did not have any effect, which suggesting that *A. artemisiifolia* assembles the *Bacillus* community in the rhizosphere soil during its invasion to facilitate its growth.

Nutrient acquisition capacity is one of the important factors that affect the competitive ability of invasive species (Daehler 2003; González et al. 2010; Sardans et al. 2016). Plant nutrient acquisition capacity is generally influenced by two factors, namely, (i) the concentrations of available nutrients in the soil and (ii) the ability of plants to absorb nutrients (Du et al. 2020). The ability of plants to absorb nutrients is an important factor in competitive plant growth. *A. artemisiifolia* and *S. viridis* belong to Asteraceae and Poaceae families, respectively, and they have different carbon fixation and nutrient absorption abilities. When *A. artemisiifolia* grows alongside *S. viridis* without *Bacillus* inoculation, the competition promotes the growth of *A. artemisiifolia* by increasing its photosynthetic product, nitrogen, and phosphorus absorption. However, the growth of *S. viridis* is inhibited by reducing photosynthetic product, nitrogen, and phosphorus absorption. The different abilities to absorb nutrients led to their difference in competitive ability. Meanwhile, soil nutrient availability is another key factor influencing the performance of invasive species (Funk and Vitousek 2007; Luo et al. 2019). Invasiveness of the exotic species is associated with significant changes in plant–soil elemental composition and stoichiometry (Daehler 2003; González et al. 2010). One of the invasion hypotheses proposes that the changes in soil microbial communities caused by invasive plants can result in positive plant-soil feedback by accumulating beneficial microorganisms in the rhizosphere (Inderjit and Cahill 2015). *Bacillus* species are prominent soil inhabitants that increase plant growth by various mechanisms, such as the production of growth-stimulating phytohormones, nitrogen fixation, and phosphorus solubilization mobilization (Wang et al. 2020). In the study, *Bacillus* diversity in the rhizosphere soils of *A. artemisiifolia* and *S. viridis* differed significantly. *Bacillus* diversity in the rhizosphere soil decreased with the invasion of *A. artemisiifolia*. Meanwhile, the study also showed that some specific *Bacillus* such as *B. megaterium*, *B. aryabhatai*, and *B. bingmayongensis* dominated in its rhizosphere soil. The ability of *B. megaterium* to modify soil available nutrients was demonstrated in a *B. megaterium* inoculation experiment. The increase in available nitrogen and phosphorus concentrations in the soil and the increase in nitrogen and phosphorus concentrations in plants suggest that *B. megaterium* in the rhizosphere soil of *A. artemisiifolia* increases the concentration of available nutrients in the soil. Furthermore, the higher density of *B. megaterium* in the rhizosphere soil of *A. artemisiifolia* provides more available nutrients in the soil. We posit that *A. artemisiifolia* assembles more *B. megaterium* than native *S. viridis* to increase the level of available nutrients in its rhizosphere soil.

Resource modification by plants may be self-beneficial because the competitive ability of a species is directly related to its ability to modify growth-limiting resources (Dybziński and Tilman 2007; Harpole 2006). Subsequently, these effects can promote positive or negative feedbacks (Suding et al. 2004). Some research showed that the invasive plant species might increase the concentrations of available nutrients during its invasion (Bajpai and Inderjit 2013; Sardans et al. 2017; Zhou et al. 2019). For example, Sardans et al. (2017) reported that the concentrations of soluble nitrate and Olsen-P in soils under invasive plants are 117% and 21% higher, respectively, than in soils under native plant communities. Besides, Zhou et al. (2019) found that invaded soils had higher nutrient stocks and soil

microbial biomass than uninvaded soils. Meanwhile, the decomposition of litter in the soil community can influence resource availability. For example, the litter of invasive *Ageratina adenophora* is rich in terpene. Soil microbial communities benefit from the release of nitrogen from decomposing litter (Bajpai and Inderjit 2013), which facilitates the growth of *A. adenophora*. Our findings reveal that *A. artemisiifolia* employs specific *Bacillus* species in its rhizosphere soil, different from that of the native species. The specific *Bacillus* species (e.g., *B. megaterium*) increases the levels of available nutrients. Furthermore, as the density of *B. megaterium* increased, the competition between *A. artemisiifolia* and *S. viridis* further enhanced the relative competitiveness of *A. artemisiifolia* while decreasing that of *S. viridis*. This finding implies that the increased relative abundance of *B. megaterium* in the rhizosphere soil of *A. artemisiifolia* can modify the resources and create a self-beneficial effect on *A. artemisiifolia*. We conclude that during its invasion, *A. artemisiifolia* assembles *B. megaterium* to create higher levels of the available nutrient in its rhizosphere soil than the native *S. viridis*, promoting its nitrogen and phosphorus absorption to enhance its competitive growth.

Declarations

Declarations of interest

None

Acknowledgements

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Figures

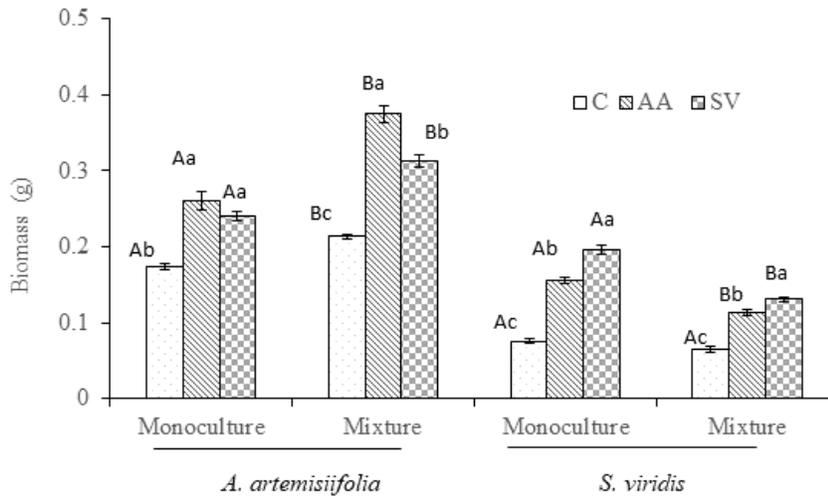


Figure 1

Effects of competition and inoculum on the biomass of *A. artemisiifolia* and *S. viridis* Notes: C, control; AA: Bacillus from the rhizosphere soil of *A. artemisiifolia* SV: Bacillus from the rhizosphere soil of *S. viridis* in the monoculture. Different higher case letters indicate significant differences between in the mixture and in the monoculture treatments at P < 0.05. Different lower case letters indicate significant differences among inoculation of different Bacillus at P < 0.05 (n=10).

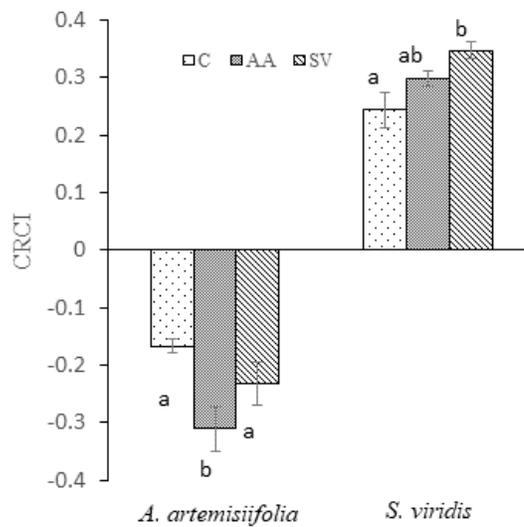


Figure 2

Effects of inoculum on CRCI of *A. artemisiifolia* and *S. viridis* Notes: C, control; AA: Bacillus from the rhizosphere soil of *A. artemisiifolia* SV: Bacillus from the rhizosphere soil of *S. viridis* . Different higher case letters indicate significant differences between in the mixture and in the monoculture treatments at $P < 0.05$. Different lower case letters indicate significant differences among inoculation of different Bacillus at $P < 0.05$ (n=10).

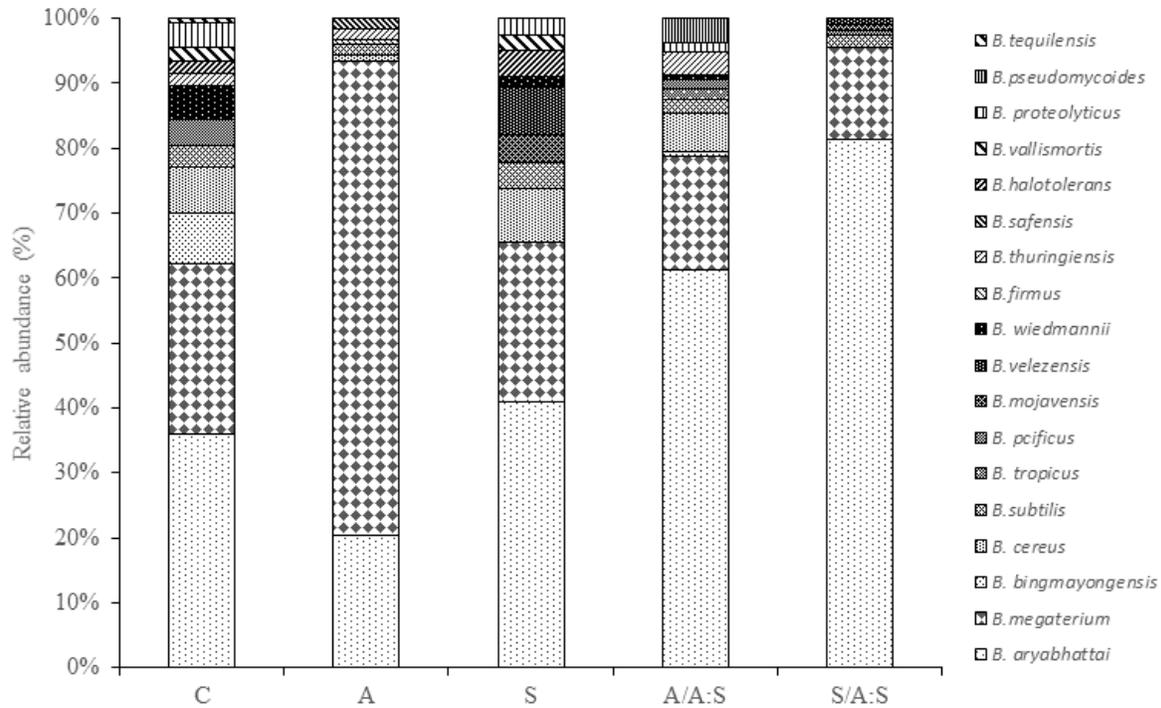


Figure 3

Relative abundance of Bacillus species in different treatments soil Notes: C, control; A: Bacillus from the rhizosphere soil of *A. artemisiifolia* in the monoculture SV: Bacillus from the rhizosphere soil of *A. artemisiifolia* in the monoculture; A/A:S, Bacillus from the rhizosphere soil of *A. artemisiifolia* in the mixture; S/A:S, Bacillus from the rhizosphere soil of *S. viridis* in the mixture (n=3).

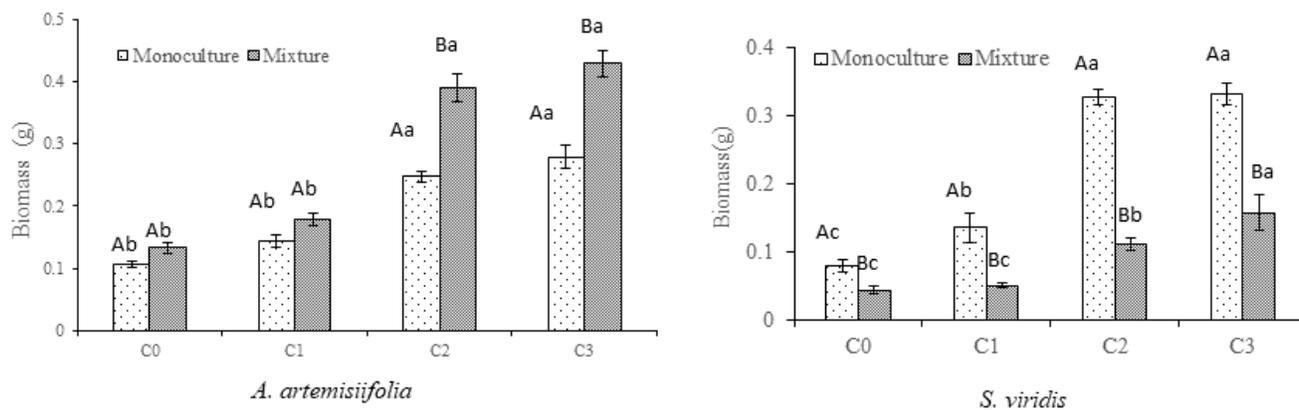


Figure 4

Effects of different concentration of *B. megaterium* on biomass of *A. artemisiifolia* and *S. viridis*. Notes: C0, control; C1: 5×10^8 cfu/mL of *B. megaterium* ; C2: 15×10^8 cfu/mL of *B. megaterium* ; C3: 30×10^8 cfu/mL of *B. megaterium*. Different higher case letters indicate significant differences between in the mixture and in the monoculture treatments at $P < 0.05$. Different lower case letters indicate significant differences among different concentrations of *B. megaterium* at $P < 0.05$ ($n=10$).

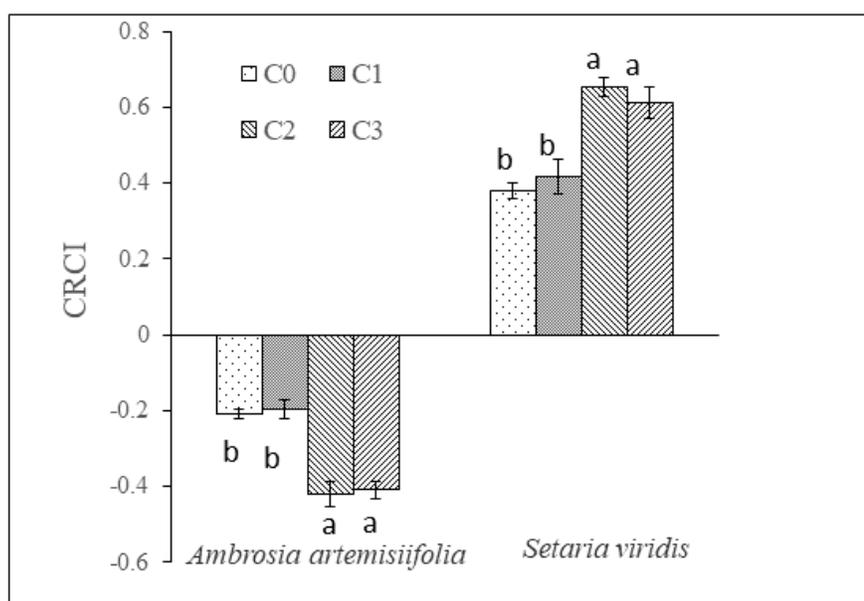


Figure 5

Effects of different concentration of *B. megaterium* on CRCI of *A. artemisiifolia* and *S. viridis* Notes: C0, control; C1: 5×10^8 cfu/mL of *B. megaterium* C2: 15×10^8 cfu/mL of *B. megaterium* C3: 30×10^8 cfu/mL of *B. megaterium*. Different lower case letters indicate significant differences among different concentrations of *B. megaterium* at $P < 0.05$. (n=10).

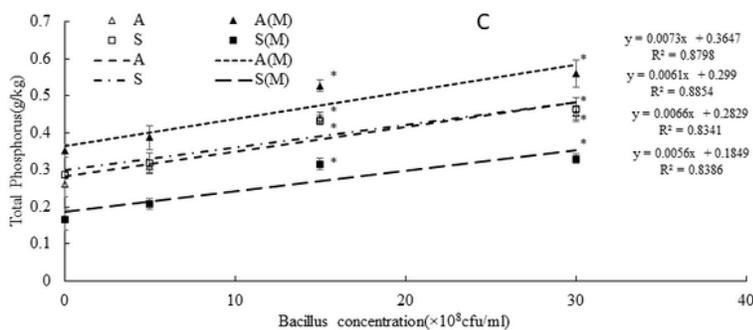
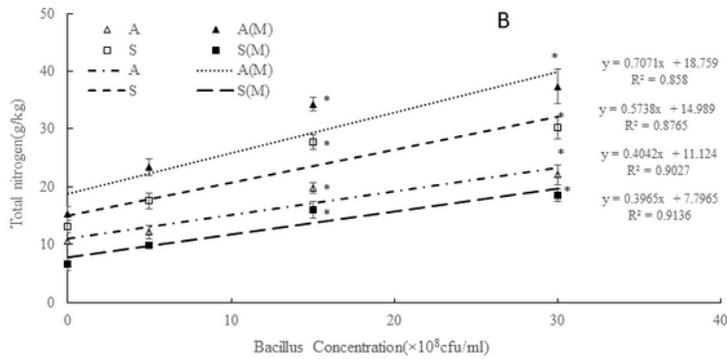
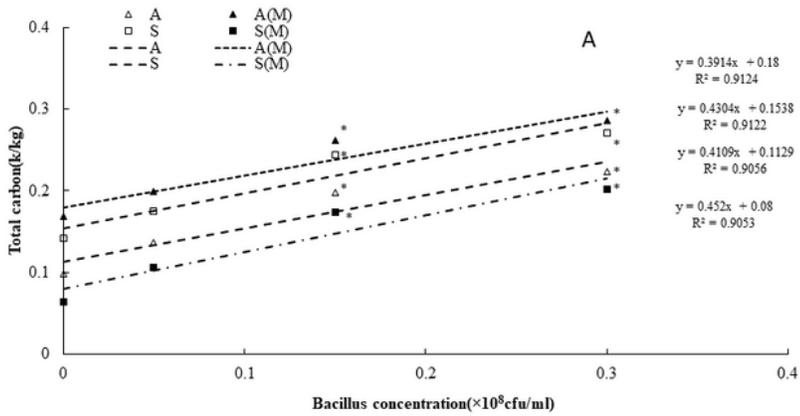


Figure 6

Effects of different concentration of *B. megaterium* on carbon(A), nitrogen(B) and phosphorus(C) levels of *A. artemisiifolia* and *S. viridis*. Notes: C0, control; C1: 5×10^8 cfu/mL of *B. megaterium* C2: 15×10^8 cfu/mL of *B. megaterium* C3: 30×10^8 cfu/mL of *B. megaterium*. Different higher case letters indicate significant differences between in the mixture and in the monoculture treatments at $P < 0.05$. Different lower case letters indicate significant differences among different concentrations of *B. megaterium* at $P < 0.05$ (n=10).

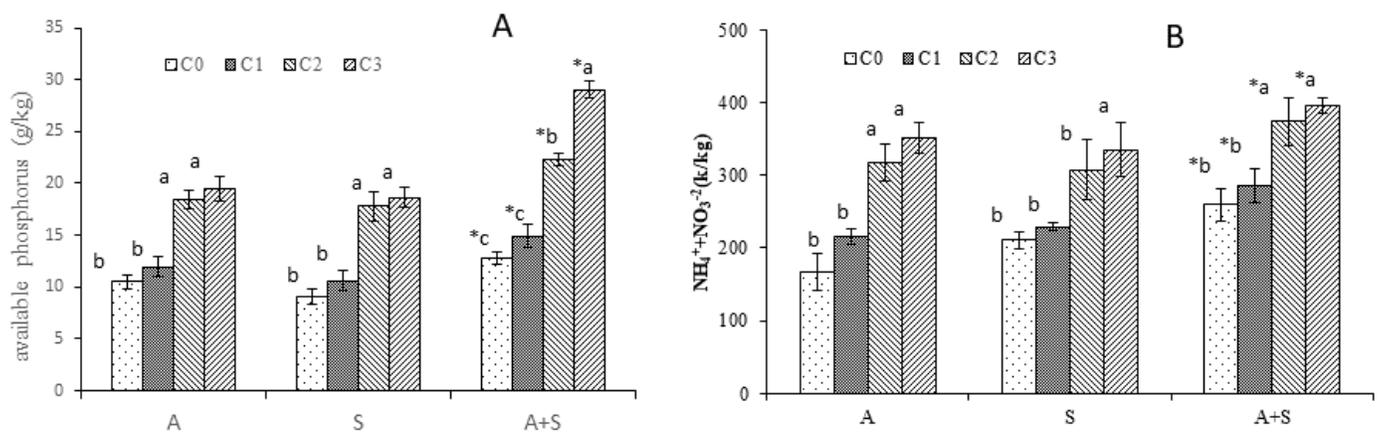


Figure 7

Effects of different concentration of *B. megaterium* on soil available phosphorus (A) and nitrogen (B) levels of *A. artemisiifolia* and *S. viridis*. Notes: C0, control; C1: 5×10^8 cfu/mL of *B. megaterium* C2: 15×10^8 cfu/mL of *B. megaterium* C3: 30×10^8 cfu/mL of *B. megaterium*. Different higher case letters indicate significant differences between in the mixture and in the monoculture treatments at $P < 0.05$. Different lower case letters indicate significant differences among different concentrations of *B. megaterium* at $P < 0.05$ (n=10).

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