Title: Tea plant-legume intercropping simultaneously improves soil fertility and tea quality by changing Bacillus species composition

Running title: intercropping with legumes in a tea garden

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Abstract

Tea plant is an economically important crop in China, but long-term monoculture and substantial chemical nitrogen fertilizer input cause soil acidification, which in turn affects the nutrient supply and tea quality. Intercropping has drawn more attention in tea gardens because this pattern is expected to improve soil fertility and tea quality and change the soil microbial community composition. However, the roles of some key microorganisms in rhizosphere soils have not been well characterized. Hereby, a “soybean in summer and smooth vetch in winter” mode was selected to investigate the effects of intercropped legumes in a tea garden on soil fertility, tea quality, and the potential changes in beneficial bacteria such as Bacillus. Our data showed that when soybeans were turned into soil, intercropping system exhibited higher soil organic matter (SOM), total nitrogen (TN), tea quality indices and the expression of Camellia sinensis glutamine synthetase gene (CsGS). Notably, intercropping significantly affected the bacterial communities and decreased the relative abundance of Bacillus but increased its absolute abundance. Bacillus amyloliquefaciens BM1 was isolated from intercropped soil and showed outstanding plant growth-promoting (PGP) properties when coinoculated with rhizobia. In winter, intercropping with smooth vetch had a beneficial effect on soil properties and tea quality. Comparably, coinoculation with strain BM1 and Rhizobium leguminosarum Vic5 on smooth vetch (Vicia villosa) showed huge improvements in SOM, TN and quality of tea leaves, accompanied by the highest level of amino acids and lowest levels of polyphenol and caffeine (p < 0.05). According to these results, our findings demonstrate that intercropping with some legumes in the tea garden is a strategy that increases SOM, TN and tea quality, and some PGP Bacillus species are optional to obtain an amplification effect.
Introduction

Tea plant (*Camellia sinensis* L.) is one of the most economically important crops in many developing countries, including China, India, Kenya and Sri Lanka. In China, there were approximately 3.10 million ha of tea plant area and 2.78 million tons of total yield in 2019. Nitrogen (N) is particularly important for tea plants because it is associated with their growth and the biosynthesis of amino acids that affect the quality properties of tea\(^1\). After the uptake of active N, the *Camellia sinensis* glutamine synthetase gene (*CsGS*) and glutamate synthase gene (*CsGOGAT*) play a vital role in transferring N to amino acids, particularly theanine\(^2\). As such, to maintain high tea yield and quality, chemical fertilizers, particularly N fertilizers, have been widely used. In some areas, the average use of nitrogen has reached up to 521 kg/ha per year\(^3\), amount that exceeds the upper limit of N fertilizer (450 kg/ha)\(^4\). There is no question about the positive effects of chemical fertilizer on increasing tea yield, however, prolonged excessive application of fertilizers often leads to environmental risks, such as soil degradation and acidification, nutrient waste, water body eutrophication and decline in tea quality\(^5\)-\(^7\). As a result, these potential problems may decrease the economic value and market competitiveness of the produced tea. Currently, considerable work has been done regarding the reduction of chemical fertilizers in tea gardens as well as the effects on tea yield and quality by low-cost and eco-friendly methods such as the application of habitat management\(^7\).

Intercropping is a useful agricultural strategy in which one plant species is grown alongside two or more crops in the same area at the same time, thereby increasing quality and output yield of crops. Compared with monoculture, intercropping improves soil fertility and plant diversity, reduces weeds, and improves light interception and utilization\(^8\)-\(^9\). Tea plants are perennial shrubs, and intercropping with other plants, such as chestnut and legumes, has been developed in a wide range of forms and
applied in different tea gardens\textsuperscript{10, 11}. In some intercropping systems, intercropped plants may compete for N from soil, hence the yield and/or tea quality may be adversely impacted. For example, Ma et al.\textsuperscript{12} found that in chestnut-tea intercropping patterns, compared with monoculture, intercropping improved the growth and quality of tea plants (including theanine content in tea leaves) but also decreased total amino acids and catechin contents. Therefore, it is particularly important for tea to select reasonable intercropped plants. Compared with other plants, legumes can obtain N from the atmosphere based on biological nitrogen fixation (BNF) when they form symbiotic relationships with N\textsubscript{2}-fixing bacteria, particularly for low-input agricultural systems\textsuperscript{9}.

Soil microorganisms play a vital role in agricultural ecosystems as they are involved in a handful of soil functions and ecological services. The soil in most tea gardens is acidic, which is due to the tea plant itself and the application of N fertilization\textsuperscript{6}. Previous studies have shown that long-term tea monoculture often has a negative effect on soil microbial diversity and beneficial microbes\textsuperscript{13, 14}, as well as soil enzymatic activity\textsuperscript{5}. For example, Li et al.\textsuperscript{15} studied the bacterial community composition from soils subjected to continuous (10- and 20-year) tea orchards and found that the relative abundance of some beneficial bacteria, such as \textit{Pseudomonas} and \textit{Bradyrhizobium}, decreased over time. On the contrary, plant species and intercropping patterns can largely affect soil properties and microbial community composition. For example, Shen and Lin\textsuperscript{16} investigated the short-term effects of soybean intercropping in a tea garden, showing that the intercropping pattern not only increased soil EC, the available P, K and some other microelements but also increased the relative abundances of Acidobacteriaceae, Burkholderiaceae, Rhodanobacteraceae and Sphingomonadaceae, which are considered as organic matter decomposers and/or plant growth-promoting bacteria.

Specifically, in agricultural systems, \textit{Bacillus}, \textit{Azobacter} and \textit{Pseudomonas} are routinely defined
as plant growth-promoting bacteria (PGPB) and directly and/or indirectly contribute to crop productivity\textsuperscript{17, 18}. In tea gardens, Arafat et al.\textsuperscript{19} found that \textit{Bacillus} and other beneficial bacteria were significantly reduced in long-term tea plantations. However, there is still insufficient information on the actual effects of intercropping systems in tea gardens on the abundance of \textit{Bacillus}. Previous studies have confirmed that coinoculation of some \textit{Bacillus} and rhizobia species has a synergistic effect on legume growth, showing higher biomasses, nodule numbers and greater levels of nitrogenase compared with single inoculation of rhizobia\textsuperscript{20, 21}. So far, we still have only a limited understanding regarding to the performance of coinoculation of \textit{Bacillus} and rhizobia on legumes in tea gardens. Nowadays, microbial community composition can be conveniently analyzed using high-throughput sequencing technologies. The predominant species in the communities are often defined using the relative abundances, however, with this approach it remains difficult to understand the dynamics of species across multiple samples\textsuperscript{22, 23}. Currently, some microbial quantification techniques, such as flow cytometry and qPCR, have been used to obtain the absolute abundance of microbial community in various environments\textsuperscript{24, 25}. Thus, combining methods based on high-throughput sequencing and qPCR will provide us further insight into the microbial community in diverse environments.

In this study, the intercropping effects of tea plants and different legume types (soybean, mung bean and smooth vetch) were examined in field experiments. Hereby, we hypothesize that tea plant-legume intercropping systems are helpful to improve soil fertility and tea quality, and this pattern will change the soil microbial community composition and enhance the counts of beneficial microorganisms such as \textit{Bacillus}. Therefore, the first objective was to estimate the effects of intercropping on both soil fertility and tea plant performance. Second, we aimed to screen some plant growth-promoting (PGP) \textit{Bacillus} species and assess their PGP effects on intercropped legumes under
both pot and field conditions. Specifically, the experimental trial was conducted in field conditions, and a conceptual model that is associated with experiment design is prepared (shown in Fig. 1). In this study, the overall objective was to provide with more information in understanding further intercropping in tea gardens.

Results

The growth performance of tea plant–soybean intercropping

In the tea garden, both soybean and mung bean could adapt the acidic conditions and exhibited adequate growth (Supplementary Fig. 1). As shown in Fig. 2a, intercropping with soybeans had no effect on soil pH regardless of all experimental periods. Compared with CK1, R1 did not show an increase in the content of SOM (Fig. 2b, \( p > 0.05 \)) but increased soil total N (TN) (Fig. 2c, \( p < 0.05 \)) in period I. In period II, SOM and TN were higher in intercropping than that in monoculture by 63.9% and 43.1%, respectively (Fig. 2b and 2c, \( p < 0.05 \)). In period I, no significant difference in polyphenol and caffeine levels between intercropping and monoculture was observed (Fig. 2d and 2e), and a decrease in the amino acid content was observed for R1 compared with CK1 (Fig. 2f, \( p < 0.05 \)). As expected, in period II, tea polyphenol and caffeine contents were significantly lower for intercropping than for monoculture by 11.6% and 23.8%, respectively (Fig. 2d and 2e, \( p < 0.05 \)), and the content of amino acids in the leaves was enhanced by 20.7% for intercropping (Fig. 2f, \( p < 0.05 \)). To examine the expressions of genes responding to the synthesis of amino acids in tea leaves, \( CsGS \) and \( CsGOGAT \) were analyzed by qPCR using gene-specific primers. The relative expression of \( CsGS \) showed a significant increase in period I and II between intercropping and monoculture (Fig. 2g). On the contrary, there was no statistical difference between the expressions of \( CsGOGAT \) in both periods (Fig. 2h). In addition, the intercropping with mung bean (\( Vignaradiata \)) in the tea garden also showed similar
patterns regarding soil properties, tea quality indices and the expression of both CsGS and CsGOGAT, as shown in Supplementary Fig. 2.

**Bacterial diversity and community composition**

A total of 826,359 high-quality sequences were obtained after sequencing and quality control. There were 33,010–59,542 valid reads obtained per sample. Overall, 5,645 OTUs were found in all soil samples. The corresponding rarefaction curves nearly tended to saturate at the selected sequencing depth (not shown). Bacterial alpha diversity indices were shown in Supplementary Table 1, suggesting that none of the soil samples significantly changed either the OTU number or the Shannon or Chao 1 indices. The beta diversity was calculated according to Bray-Curtis method at OTU level. As shown in Supplementary Fig. 3, the soil samples were separated from each other. PERMANOVA indicated that intercropping with soybean in the tea garden significantly changed the soil bacterial communities \( (p < 0.05)\).

8 bacterial phyla (relative abundance > 1%) were identified across all soil samples: Proteobacteria, Chloroflexi, Actinobacteria, Acidobacteria, Gemmatimonadetes, Bacteroidetes, Verrucomicrobia and Patescibacteria (Fig. 3a). Compared with CK1, R1 treatment showed an increase in the relative abundance of Proteobacteria from 22.3% to 43.0% and decreases in Chloroflexi and Acidobacteria from 25.0% to 7.0% and 20.1% to 8.8%, respectively. In period II, intercropping enhanced the relative abundances of Proteobacteria and Acidobacteria from 18.2% to 23.6% and from 8.9% to 19.5%, respectively, and decreased the relative abundances of Chloroflexi and Actinobacteria from 35.6% to 27.1% and from 23.6% to 12.9%, respectively. Furthermore, the dominant genera of each sample (top 15) were also assessed (Fig. 3b): AD3_norank, Gaiellales_norank, HSB OF53-F07, Acidobacteriales_norank, Acidothermus, subgroup 2, Gemmatimonadeceae_uncultured,
Elsterales_norank, Aeromonas, Xanthobacteraceae_uncultured, Gemmatimonas, Saccharimonadales_norank, subgroup 6, Bradyrhizobium and Acidibacter. Volcano plots were employed to show the difference between pairs of soil samples (Fig. 3c and 3d). Compared with CK1, R1 treatment significantly increased the relative abundances of Saccharimonadales_norank, Chitinophagaceae_uncultured, 67–14, Sphingomonadaceae and rhizobia (Allorhizobium, Neorhizobium, Pararhizobium and Rhizobium), accompanied by lower relative abundances of Acidobacteriales_norank, Subgroups 2 and 13, Elsterales_norank, Candidatus Udaceobacter and JG30-KF-AS9 (p < 0.05). Compared with CK2, the R2 treatment significantly enriched Haliangium and ADurb. Bin063–1 and decreased the relative abundances of FCPS473, Gaiellales_norank and HSB OF53-F07.

Accordingly, a special focus was given to the taxonomy of Bacillus because of their ecological importance. The relative abundance of Bacillus in each sample was compared (Fig. 3e), and the results showed that intercropping with soybean decreased the relative abundance of Bacillus in both periods. As shown in Fig. 3f, in period I, intercropping decreased the absolute abundance of Bacillus (p < 0.05). However, in period II, a different pattern was observed, showing that, compared with monoculture, intercropping significantly increased the copy number of Bacillus in the tea garden (p < 0.05). These results were further supported by the total number of bacteria and spores detected using the traditional dilution-plate method (Supplementary Table 2).

Isolation of Bacillus species and their effects on the growth and nodulation of legumes

9 (B6, B8, B12, B19, B24, B42, B44, BM1 and BM10) and 7 (CB6, CM1, CM6, CM8, CM9, CM14 and CM15) Bacillus species were obtained from the CK2 and R2 soil samples, respectively. The symbiotic performance of each Bacillus species and Br. diazoefficiens USDA110 was conducted on
soybeans (Supplementary Fig. 4). The data showed that among all *Bacillus* species, only strain BM1, identified as *Bacillus amyloliquefaciens*, showed an improvement in both nodule number and nitrogenase activity, exhibiting proud PGP properties. To confirm the effects of strain BM1 on the performance of cold-tolerant legumes (smooth vetch and *Astragulus sinicus* L.), pot experiments were conducted in the laboratory (Fig. 4). The results suggested that, compared with single inoculation, coinoculation with BM1 and Vic5 significantly promoted the height of smooth vetch, dry weight, number of nodules and activity of nitrogenase by 20.8%, 32.0%, 54.1% and 16.5%, respectively. A similar trend was observed for coinoculation with BM1 and Mh93 on the growth of *Astragulus sinicus* L. by 13.9%, 22.6%, 30.1% and 10.8%, respectively.

**Strain BM1 improved the efficiency of the tea plant-SV intercropping system in winter**

Based on the biomass of smooth vetch and *Astragulus sinicus* L. (Fig. 4), smooth vetch was selected as an intercropped plant in the garden during the winter. As shown in Fig. 5, compared with single inoculation, coinoculation significantly improved the fresh weight of smooth vetch in the tea garden by 11.3% (Fig. 5a, *p* < 0.05). Similarly, the activity of nitrogenase was also enhanced in the coinoculation treatment by 14.8% (Fig. 5b, *p* < 0.05). In contrast, planting of smooth vetch in the tea garden could effectively enhance soil pH values, regardless of inoculation mode and sampling time (Fig. 5c). Compared with CK3, R3 and RB3 treatment did not affect the accumulation of SOM. However, in period IV, R4 and RB4 did (Fig. 5d, *p* < 0.05). Compared with the corresponding CK, R3 and RB3 treatments significantly increased the levels of TN by 23.4% for R3 and 22.1% for RB3 treatments in period III and by 47.6% for R4 and 52.4% for RB4 treatments in period IV, and a striking increase in TN also appeared in period IV for the inoculated treatments compared with that from period III (Fig. 5e, *p* < 0.05). Additionally, intercropping systems had different effects on tea quality indices.
Compared with the corresponding CK, the RB3 and RB4 treatments decreased the content of tea polyphenols in both period III and IV, respectively with the minimum value obtained in period IV (Fig. 5f, p < 0.05). Intercropping did not affect the caffeine content in period III, but decreased it in period IV (Fig. 5g, p < 0.05). In contrast, intercropping increased the content of amino acids in the leaves in both period III and IV, but particularly in period IV (Fig. 5h, p < 0.05). Similarly, the coinoculation treatment showed a higher content of amino acids compared with that from the single inoculation treatment (p < 0.05).

Compared with CK4, single inoculation of R. leguminosarum Vic5 (R4 treatment) increased the total number of bacteria but not the spores or gene copies of Bacillus (Table 1). On the contrary, coinoculation with Vic5 and BM1 significantly increased all of above compared with all other treatments (p < 0.05). Accordingly, in period IV, 27, 27 and 24 Bacillus species were randomly isolated from the CK4, R4 and RB4 treatments, respectively (Supplemental Table 3), showing more Bacillus species in the RB4 treatment. Soybeans were meant to be planted in the same garden for the next intercropping cycle (soybean + smooth vetch), therefore, the PGP effects of these strains on the performance of soybeans were assessed when they were coinoculated with Vic5. The results suggested that 6/27, 10/27 and 14/24 strains could efficiently improve both the growth of soybeans and symbiotic nitrogen fixation efficiency (Supplementary Fig. 5). Based on statistical analysis, compared with CK4, R4 treatment did not increase the ratio of PGP Bacillus, but RB4 treatment significantly did.

**DISCUSSION**

Intercropping is an important agricultural technique that has been applied in China since ancient times. Previous studies have demonstrated that intercropped plants in tea gardens, such as Chinese chestnut, fruit and soybean, are beneficial for improving soil nutrients and tea quality. In this study,
legumes were selected as intercropped plants, demonstrating their potentials in tea gardens. One important reason is that legumes will not compete with other plants for N nutrients because they can obtain the necessary N from the atmosphere based on BNF. Another advantage is that legumes can be rapidly decomposed when they are turned into soil and increase microbial counts and soil nutrients\textsuperscript{16, 27}. This opinion was previously confirmed by Farooq et al.\textsuperscript{9}, who examined the intercropping of peanut-tea and found that intercropping actively enhanced soil fertility and positively impacted soil health. In this study, our data suggested that intercropped soybean and smooth vetch could increase the content of soil TN (Fig. 2c and Fig. 5e), which was in accordance with previous studies. N is necessary for plant growth and amino acids synthesis. In a very recent study conducted by Duan et al.\textsuperscript{28}, who investigated the effects of intercropping with soybean on the secondary metabolites of tea plants and found that intercropping could particularly promote amino acids synthesis when the soybean was in profuse flowering stage. This observation could partly support the finding of this study, and help us explain the physiological changes of the tea plants.

In addition, our data also suggested that intercropping with either soybean or mung bean was helpful to increase the level of SOM, particularly in period II (Fig. 2b and Supplementary Fig. 2b), suggesting that legume maturation in soils was extremely important to increase soil fertility. It is worth noting that SOM in soil is usually stable and changes slowly. However, compared with CK2, a significant increase of SOM was observed in R2 treatment from 13.6 g/kg to 22.3 g/kg. The increase likely attributed to a signal bias from legume residues in soil that were oxidized by K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}-H\textsubscript{2}SO\textsubscript{4} using our selected method, and hence, measured values of SOM were greater than the true values. Thus, continuous application of intercropping in the same gardens should be maintained to obtain more information. In tea gardens, soil acidification can result in accumulation of aluminum and a lack of
phosphorus, potassium and magnesium. In this study, we found that soil pH increased (by approximately 0.9 units) after intercropping with smooth vetch, suggesting that this intercropping pattern was beneficial for the soil chemical environments. However, in a different work (yet unpublished), data showed that soil pH would decrease after intercropping. Therefore, a long-term intercropping experiment would be crucial to precisely simulate this phenomenon. In Nanjing, tea leaves are usually picked around China’s Tomb Sweeping Day (the 5th of April). Our data showed that after the intercropping mode “soybean + smooth vetch”, tea leaves in period IV achieved the highest quality during the tea-picking season, with the highest content of amino acids and the lowest contents of tea polyphenol and caffeine. Therefore, according to the above, we concluded that long-term application of intercropping with legumes in tea gardens, as well as the application of beneficial Bacillus species, is a beneficial strategy to maintain high soil fertility tea quality.

High-throughput sequencing data revealed that Proteobacteria, Chloroflexi, Actinobacteria and Acidobacteria were the top 4 phyla in the tea garden, which was in line with the findings of Fu et al. and Shen and Lin. An obvious increase in the relative abundance of Proteobacteria in the flowering-podding period of soybeans was also observed. It is well known that plants need enough N during the flowering period. Some studies have indicated that a wide range of N$_2$-fixing bacteria belong to Proteobacteria. According to these results, we hypothesized that soybean utilized these special bacterial groups during the flowering period to help themselves obtain the necessary N. At genus level, intercropping increased the relative abundance of rhizobia (Allorhizobium, Neorhizobium, Pararhizobium and Rhizobium), which could plausibly support the above assumption. Compared with monoculture, intercropping with soybean significantly increased the relative abundance of Haliangium in period II (Fig. 3d). Previous studies have suggested that Haliangium has PGP effects. A recent study
conducted by Wang et al.\textsuperscript{33} highlighted that legume straw (\textit{Chamaecrista rotundifolia}) combined with chemical fertilizers and poultry manures increased the relative abundance of \textit{Haliangium} in acidic tea soils, which accounted for 1.56\% of the total bacteria. Hence, intercropping with soybean in tea gardens can promote specific bacterial populations. In period II, bacterial groups had increasing and/or decreasing trends (Fig. 3d), however, only a few of them could be identified to the genus or species, which made it difficult for us to understand the ecological roles of these bacterial members.

\textit{Bacillus} species are widely distributed in agricultural soils and are associated with biological control agents of biotic diseases and plant growth, quality improvement and yield increase\textsuperscript{34, 35}. In tea gardens, \textit{Bacillus} species are frequently obtained from the soil samples and root surface because of their acid-resistant and endogenous spore-forming characteristics\textsuperscript{36}. In this study, our data suggested that both the relative and absolute abundances of the \textit{Bacillus} in period I decreased after intercropping with soybean, but an inconsistent trend was observed for period II, showing a decreased relative abundance but an increased absolute one in the intercropping system. Although the relative abundance of the microbial communities provides important insights into the diversity and microbial community composition, it does not reflect the absolute abundance of each microbial group\textsuperscript{37}. Previous investigations observed both similar and opposite results with respect to the relative and absolute abundances\textsuperscript{24, 25, 38}. Thus, combining the relative and absolute abundances can accurately indicate the changes of microbial communities. According to these results, we hypothesized that organic amendments (soybean straw) in soils promoted the multiplication of \textit{Bacillus}, which are involved in the decomposition of organic matter. This assumption was partially supported by Su et al.\textsuperscript{39}, who investigated the effects of long-term organic fertilization on the soil active bacterial communities using high-throughput sequencing and qPCR-based SmartChip assays and found that organic fertilization
dramatically enhanced the abundance of *Bacillus*. Similarly, Feng et al.\textsuperscript{40} reported the effect of organic manure fertilization on agroecosystems, showing that *Bacillus asahii* responded to organic manure fertilization and become the dominant species after 2-4 years. In the present study, the increased absolute abundance of *Bacillus* in the R2 treatment in the present study thus suggests that intercropping with soybean could accelerate soil carbon cycle in the tea garden, which in turn could have been beneficial to the tea quality.

*Br. diazoefficiens* USDA110 is commonly recognized as a highly efficient N\textsubscript{2}-fixing strain when it forms symbiotic relationships with soybeans. Our data clearly demonstrated that coinoculation of soybean with *Bacillus* species and USDA110 altered the growth performance of legumes and significantly improved nodulation and BNF (Supplementary Fig. 4), and these effects were also observed in combinations of BM1 and Mh93 on *Astragalus sinicus* L. and BM1 and Vic5 on smooth vetch (Fig. 4). The obtained data suggested that some PGPR could positively interact with rhizobia and/or plants and then improve the growth performance. Similar findings were obtained by Korir et al.\textsuperscript{20}, who examined the coinoculation effect of rhizobia and PGPB on common bean growth, showing that coinoculation of IITA-PAU987 and *Bacillus megaterium* significantly increased the nodule weight and biomass compared with IITA-PAU alone in a low-phosphorus soil. A study investigated by Tilak et al.\textsuperscript{41} suggested that coinoculation of pigeonpea with *Rhizobium* and either *B. cereus*, *Pseudomonas putida* or *P. fluorescens* contributed to a significant increase in plant growth, nodulation and enzyme activity compared with single and uninoculated controls. Under saline-alkali conditions, Han et al.\textsuperscript{42} obtained 278 *Bacillus* species from soils and found that *Bacillus cereus* could promote the growth of sinorhizobia and alleviate the effects of saline-alkali stresses on nodulation, suggesting a key role of *Bacillus* species in shaping rhizobia-host interactions in soybean. These results suggested that
coinoculation with rhizobia and PGPR could improve the growth performance of legumes and confirmed their applicability in agriculture. In this study, among all *Bacillus* species, *B. amyloliquefaciens* BM1 was considered the most efficient strain in improving plant performance based on the growth, nodulation and activity of nitrogenase, which was in accordance with previous studies. This strain has been previously recorded as a PGPR that enhanced soybean nodulation when coinoculated with *Bradyrhizobium japonicum*, and this improvement is ascribed to the production of auxin, gibberellins and salicylic acid. In a recent study, Sibponkrung et al. identified the synergistic effects between *B. velezensis* S141 and *Br. diazoefficiens* USDA110 on nodule growth and N₂ fixation, revealing that IAA and cytokinin produced by S141 promote USDA110 to determine larger nodules. In addition to phytohormones, Rajendran et al. suggested that *Bacillus* strains NR4 and NR6 could mediate the growth of *Rhizobium* sp. IC3123 and increased nodule number by producing siderophores.

In our study, strain BM1 showed synergistic effects with rhizobial strains (USDA110, Mh93 and Vic5) and/or legumes, which might be due to the production of some unknown metabolites. In addition, our data also suggested that intercropping with both strains BM1 and USDA110 led to an increase in the ratio of PGP *Bacillus*, which was in turn beneficial to the sustainable development of tea gardens. However, the interactions among PGPR, rhizobia and legumes are extremely complex, and the process may be affected by other factors such as different soil nutrient status, soil microorganisms and/or some unknown conditions. Therefore, further investigations are needed to further expand our understanding of this field.

**Conclusion**

In summary, our study investigated the effects of legumes as intercropped plants in a tea garden and the results obtained confirmed that the “soybean in summer and smooth vetch in winter” mode is...
beneficial for increasing soil fertility and improving tea quality. In such intercropping systems, PGP 

*Bacillus* species can act as biostimulants and operate as inocula to improve the growth performance of legumes and tea plants.

**Materials and Methods**

**Tea – legume intercropping in field experiment**

The field experiment was conducted at Jiangsu Bocha Tea Industry Co. Ltd., Nanjing, China. The location has a northern semitropical monsoon with an average annual temperature, rainfall and frost-free period of 15.7 °C, 1072.9 mm and 224 days, respectively. The examined tea garden has been subjected to monoculture management for approximately 20 years. For the field experiments, a randomized complete block design with two treatments was conducted in the field experiments. Each treatment contained three rows of tea plants (each row: 15 m × 3 m = 45 m²). Monoculture was used as the control (CK), and soybean was planted as the intercropped plant. For inoculation, bacterial inoculum was prepared according to the following process: *Br. diazoefficiens* USDA110 was cultured using tryptone yeast medium (TY) at 28 °C for 5 days (180 rpm). Bacterial cells were obtained by centrifugation (6,000 g for 10 min), and then re-suspended and precipitated five times in sterile distilled water, and ultimately preserved in ddH₂O. Bacterial density was diluted to approximately 10⁸ cells/mL.

Before sowing, bacterial inoculation of *Br. diazoefficiens* USDA110 was applied to seed in the form of slurry. Specifically, slurry enriched with bacterial communities and soil were thoroughly mixed according to the following ratio: 200 g seeds, 200 g soil, 10 mL bacterial suspension and 40 mL water.

Soybean (*Glycine max*) of a ‘Hefeng47’ variety was sown in late May 2019 (45 kg/ha) and flowered and/or podded in early Jun 2019, and this period was defined as period I. Then, the soybeans were turned into soil until late August 2019 (for approximately 40 days), and this period was defined as
period II. The conceptual model that was associated with the experimental design and sampling periods is shown in Fig. 1. In monoculture treatment, the weeds were cut aperiodically, and the residues were turned into soil accordingly. In intercropping rows, no weed or only a few weeds were present as plantation was almost-to-completely controlled by legumes. All other management aspects between monoculture and intercropping were the same. Soil and tea leaf samples were collected from the two covering patterns in period I (CK1 and R1, respectively) and period II (CK2 and R2, respectively), respectively. At the same time, mung bean of ‘Sulv4’ variety was also used as intercropped plant in the tea garden following the above mentioned method.

**Plant performance and soil analysis**

The plant height of each plant in the pot experiment was recorded using a ruler. The nodule of each legume plant was manually counted, and the activity of nitrogenase was determined using an acetylene reduction assay according to Ning et al. Catechin and caffeine contents were analyzed using HPLC (Waters, USA) according to national standards (GB/T8313–2008), while the content of amino acids was determined according to ninhydrin colorimetry method (GB/T8314–2013). Soil samples were collected from the junctional zones (5-15 cm) of the roots of both tea plants and soybeans (period I) and roots of tea plant and legume residues (period II). Obvious plant roots and residues were carefully removed. Soil samples were immediately transported to the laboratory. Each soil sample was divided into three parts. One part was deposited at -80 °C for extraction of bacterial DNA and RNA, the second part was stored at 4 °C for cell counting and microbial screening, and the third part was air-dried in the laboratory for soil chemical analysis. Soil pH was measured using a pH meter (PHS-3CT) in a 1:2.5 soil/water ratio. SOM was analyzed for 0.5 g soil with 10 mL of 0.136 mol/L K$_2$Cr$_2$O$_7$-H$_2$SO$_4$, with 3-4 drops phenanthroline indicator, and titrated with 0.2 mol/L standard FeSO$_4$.
solution. TN was measured for 0.5 g soil with 2 g accelerator (K₂SO₄: CuSO₄: Se (w : w : w) = 100:10:1) and 5 mL of H₂SO₄ (overnight). Soil samples were heated using a boiling furnace (200 °C for 15 min, 380 °C for 3-4 h) until the color turned into off-white, and incubation was further maintained for another 1 h. The liquid was diluted with ddH₂O to a volume of 100 mL. A continuous flow analytical system (San++ System, Skalar, Holland) was used to measure TN.

Validation of the nitrogen metabolism-related genes CsGS and CsGOGAT

Total RNA in tea leaves was extracted using a RNA kit (RNA Simply Total RNA kit; Tiangen, Beijing, China). cDNA was then synthesized using the PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan) according to the manufacturer’s protocol. The primers of CsGS were F: 5’-GCC AAT CCC AAC AAA TAA GAG G-3’) and R: 5’-TAT CCG CAC CAA TAC CAC AG-3’, and the primers of CsGOGAT were F: 5’-CGA AAA ACG GTG ACA GA T G-3’) and R: 5’-AGG AAG AGC GAC GAG AAT G-3’. qPCR was performed in a 20 μL reaction mixture that contained 80–100 ng cDNA, 200 nM of each primer and 10 μL LightCycler 480 SYBRGREEN I Master Mix (Roche, Basel, Switzerland). All reactions were performed in duplicate in 96-well plates. Real-time PCRs were conducted according to the following conditions: 95 °C for 30 s, 95 °C for 30 s and 40 cycles at 95 °C for 10 s and 60 °C for 30 s. Following melting curve analysis at 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s, β-actin expression was used as an internal control. Finally, the expression levels were analyzed using the 2^ΔΔCt method for each sample.

DNA extraction, PCR amplification and sequencing

Soil DNA samples were extracted from approximately 0.5 g of soil using a FastDNA SPIN Kit for Soil (MP, Biomedicals) following the manufacturer’s instructions. Primer set 338F (5’-ACT CCT ACG GGA GGC AGC AG-3’) / 806R (5’-GGA CTA CHV GGG TWT CTA AT-3’) with barcode was
employed to amplify bacterial V3-V4 hypervariable regions of the 16S rRNA gene. PCR amplification was conducted according to Huang et al.\textsuperscript{48} After DNA purification (E.Z.N.A. Cycle Pure Kit, OMEGA) and quantification (NanoDrop-2000), PCR products were sent to Biozeron Bio-Technology Co., Ltd. (Shanghai, China), and sequencing was carried out via the Illumina MiSeq\textsuperscript{TM} System platform.

**Isolation of Bacillus spp. from tea–soybean intercropping soil**

*Bacillus* species were isolated from soil samples according the following procedure. Briefly, 10 g soil was added to an Erlenmeyer flask that contained 90 ml sterilized water. The Erlenmeyer flask was then successively shaken (180 rpm) for 20 min and heated in a water bath (80 °C) for 15 min. Soil suspensions were serially diluted in a 10-fold gradient (10\textsuperscript{-2}, 10\textsuperscript{-3} and 10\textsuperscript{-4}). Then, 0.1 mL of each soil suspension was inoculated onto minimal medium (MM) containing the following (per L) mannitol (10 g), yeast powder (0.5 g), K\textsubscript{2}HPO\textsubscript{4} (0.5 g), MgSO\textsubscript{4} (0.097 g) and NaCl (0.1 g). The plates were placed in an incubator for 2–3 days (30 °C), and then the colonies were randomly selected from the plates. The strains were purified streaking on fresh medium 2-3 times and identified based on their 16S rRNA gene sequences.

**Quantification of Bacillus by qPCR**

qPCR was conducted using a Roche Light Cycler 480 system. The primer set BacF/R1378\textsuperscript{49} was used for the quantification of *Bacillus* with SYBR green-based reactions (three replications). The 20 μL reaction mixture contained 0.25 μL template DNA, 200 nM of each primer and 10 μL LightCycler 480 SYBRGREEN I Master Mix (Roche, Basel, Switzerland). Reactions were run for 5 min at 95 °C, 45 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, followed by plate reading. Melting curve analysis and electrophoresis were used to check the amplification specificity. A qPCR standard was generated using plasmid DNA from one clone containing each of the above genes. A series of 10-fold
dilutions of standard template were used per assay. The $R^2$ value for the standard curves was 0.99, and the qPCR efficiency was $\geq 0.98$ for the quantitative assays.

**The effects of Bacillus spp. on nodulation and N$_2$ fixation of legumes**

*Br. diazoefficiens* USDA110 was cultured as described above. *Bacillus* spp. were cultured in TSB and washed accordingly. Each *Bacillus* strain population was then adjusted to approximately $10^8$ CFU/ml. For the nodulation assays, sterilized soybeans were planted in glass tubes (parceled with black papers) containing Fahraeus medium and grown in controlled conditions (16 h light/8 h darkness, 30 °C). Cells of USDA110 (1 mL) were inoculated into the roots of soybean seedlings when their roots reached approximately 2–3 cm. For coinoculation, liquid volume (1 mL) that contained an equal amount of USDA 110 and each of the *Bacillus* strain solutions was added to the roots of the soybean seedlings. N-free Hoagland’s solution was used to supply with the necessary elements. After 4 weeks, the growth of soybean seedlings was recorded, and nodulation and the associated nitrogenase were analyzed accordingly. Using the same method, the effects of coinoculation of the most efficient *Bacillus* with *Rhizobium leguminosarum* Vic5 on smooth vetch or with *Mesorhizobium huakuii* Mh93 on *Astragalus sinicus* L. were confirmed after 6 weeks.

**Strain BM1 enhanced the performance of tea-smooth vetch intercropping**

To enhance the effects of intercropping in the tea garden, smooth vetch was selected as an intercropped legume due to its cold resistance and larger biomass yield during the winter. In this experiment, all treatments were conducted in the same field area. In addition, the soybean rows planted in summer were divided into two parts that were used for single and dual inoculations (Fig. 1). Therefore, there were overall three treatments: monoculture, intercropping smooth vetch with Vic5 only and coinoculation with Vic5 and BM1. The seedlings, bacterial suspension and inoculation
method were performed as described above. The seeds of smooth vetch were sown in mid-Oct 2020 (45 kg/ha), and the smooth vetches were turned into soils in early Apr 2021 (flowering–podding period, period III) for approximately 30 days (early May 2021, period IV). Soil and tea leaf samples were collected in periods III and IV. Soil properties, plant growth parameters, total amino acids content, tea polyphenols and caffeine in tea leaves, nodulation and the activity of nitrogenase were all determined as mentioned above.

**Data analysis**

The data obtained by Illumina sequencing were analyzed according to QIIME. Briefly, raw sequences were separated based on their unique barcodes, and the barcode and primer sequences were then removed. The reads were clustered into OTUs (operational taxonomic units) based on 97% sequence similarity. Finally, a representative sequence for each OTU was assigned to sequences deposited in the SILVA database (v132). The relative abundance of each bacterial group and alpha diversity indices, including OTU number, Chao 1 and Shannon, were analyzed with QIIME according to the tutorial. STAMP (version v2.13) was used to analyze the differences of bacterial groups in the relative abundances between each pair of samples. Nonmetric multidimensional scaling (NMDS) was conducted to explore the differences in bacterial communities based on Bray-Curtis distances using the R vegan package. Permutational multivariate analysis of variance (PERMANOVA) was performed to verify whether the bacterial communities showed significant differences among soil samples (R package vegan). One-way ANOVA and Fisher’s least significant difference (LSD) post hoc test (Bonferroni method) were employed to determine the differences among soil samples using R (version 4.0), while the differences between two samples were confirmed using t-test analysis.

**Data availability**
The authors declare that all data in this study are available within the paper and its supplementary files.

The raw sequence data reported in the paper have been deposited in the Genome Sequence Archive (https://bigd.big.ac.cn/gsa/) under accession number CRA005114.

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Author contributions

Z.H., W.F. and Z.Z. conceived and designed the project. Y.C., S.H., W.W and C.C. performed the experiments. Y.C., C.C., J.D., X.C., W.W., H.W. and Y.D. analyzed the data. Z.H. and C.C. wrote the manuscript. Z.H., E.P., L.Z., W.F. and Z.Z. discussed and revised the manuscript.

Conflict in interest

The authors declare that they have no conflict of interest.

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### Table 1 Number of bacteria and spores in different treatments

| Sample | Number of bacteria ($\times 10^7$ CFU/g soil) | Number of spores ($\times 10^5$ CFU/g soil) | Copies of *Bacillus* ($\times 10^3$/g soil) |
|--------|-------------------------------------------|-------------------------------------------|---------------------------------------------|
| CK4    | 1.06±0.22$^c$                            | 2.33±0.58$^b$                             | 3.68±0.22$^b$                              |
| R4     | 2.17±0.04$^b$                            | 4.00±1.73$^b$                             | 3.05±0.36$^b$                              |
| RB4    | 2.96±0.81$^a$                            | 16.67±1.15$^a$                            | 10.60±0.94$^a$                             |

Different letters mean significant differences ($p < 0.05$). CK4, monoculture in period IV; R4, intercropping with smooth vetch and inoculation with USDA110 in period IV; RB4, intercropping with smooth vetch and inoculation with USDA110 and BM1 in period IV.
Figure legends

Fig. 1 The conceptual model of the experimental design in this study. In summer, soybean was selected as intercropped plant, and *Bradyrhizobium diazoefficiens* USDA110 was inoculated as rhizobia (R) that formed nodules on soybean. The period I meant that soybeans were flowering and/or scabbing, and period II meant that soybeans were turned into soils for approximately 40 days. Monoculture was used as control (CK). In winter, smooth vetch and *Rhizobium leguminosarum* Vic5 were selected as intercropped plant and related rhizobia, respectively. In addition to monoculture and single inoculation, coinoculation with rhizobia and *Bacillus amyloliquefaciens* BML was also assessed in field conditions (RB). Similarly, period III meant smooth vetches were flowering and/or scabbing, and period IV meant that smooth vetches were turned into soils for approximately 30 days.

![Fig. 1](https://example.com/fig1.png)
Fig. 2 The effects of monoculture and intercropping (soybean-tea plant) under field conditions. a soil pH. b soil organic matter. c total nitrogen. d tea polyphenol in tea leaves. e caffeine in tea leaves. f amino acids in tea leaves. g the relative expression of CsGS. h the relative expression of CsGOGAT. I and II represented period I and II, respectively. CK1 and CK2, monoculture of tea plant in period I and II, respectively; R1 and R2, intercropping with soybean in the tea garden in period I and II, respectively. All data were shown as the mean ± SD (n=3). Different letters represent significant differences (p < 0.05).

Fig. 3 Intercropping changed the soil bacterial community composition in the tea garden. a Relative abundance of major bacterial communities (> 1%). b Top 15 genera of each soil sample. c and d Differences in bacterial groups at the genus level between R1 and CK1 and R2 and CK2, respectively. The data were determined using STAMP with two-group comparisons. The effect size represents the difference in the relative abundances of some bacterial genera between two samples. The red and blue dots indicate the samples with p values < 0.05 by Welch’s t-test and an effect size ≥ 0.5 or ≤ -0.5, while the gray dots mean the samples with p values > 0.05 or effect size ranging from -0.5 to 0.5. The red, blue and gray dots indicate increased, decreased and unchanged bacterial groups in relative abundances,
respectively. e and f, Relative and absolute abundance of Bacillus, respectively. I and II represented period I and II, respectively. CK1 and CK2, monoculture of tea plant in period I and II, respectively; R1 and R2, intercropping with soybean in the tea garden in period I and II, respectively. *, p < 0.05; **, p < 0.01.

Fig. 4 The effects of Bacillus amyloliquefaciens BM1 on the performance of smooth vetch and Astragalus in pots. a height of legumes. b dry weight of legumes. c number of nodules. d the activity of nitrogenase. Vic5, Rhizobium leguminosarum Vic5; Mh93, Mesorhizobium huakuii Mh93. *, p < 0.05; **, p < 0.01.
Fig. 5 Effects of intercropping with smooth vetch on soil fertility and secondary metabolites in tea leaves with field experiments. a Fresh weight of smooth vetch at 6 weeks postinoculation. b the activity of nitrogenase in single and dual inoculation. c soil pH. d soil organic matter. e total nitrogen. f tea polyphenol. g caffeine. h amino acids. Different letters represent statistic differences ($p < 0.05$). III and IV represented period III and IV in Fig. 1, respectively. CK3 and CK4, monoculture in period III and IV, respectively; R3 and R4, intercropping with smooth vetch and inoculation with USDA110 in period III and IV, respectively; RB3 and RB4, intercropping with smooth vetch and inoculation with USDA110 and BM1 in period III and IV, respectively.