Linking microbial inoculants and associated changes in the soil microbiome to plant-soil performance in degraded lands

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Linking microbial inoculants and associated changes in the soil microbiome to plant-soil performance in degraded lands

Koppeling van microbiële inoculatie en de bijbehorende veranderingen in het bodemmicrobioom aan plant-bodemprestaties in gedegradeerde gebieden

(met een samenvatting in het Nederlands)

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Contents

Chapter 1 General Introduction

1. Land degradation and applications of soil beneficial microorganisms

The increasing demand for food has led to intensive farming and unsustainable management of agricultural lands with excessive chemical inputs, resulting in serious land degradation worldwide (Tilman et al. 2011, Kraaijvanger and Veldkamp 2015). Land degradation is typically characterized by reduction in soil fertility (Rashid et al. 2016), soil erosion, decline of biological productivity (Kraaijvanger and Veldkamp 2015) and biodiversity loss both underground (Araujo et al. 2013) and aboveground (Newbold et al. 2015). To restore soil function and land productivity, efforts to reverse the loss of soil fertility, plant productivity, and microbial functional diversity have received increased attention in recent years. Conventional restoration practices, such as applications of inorganic and organic fertilizer, have been linked with negative environmental impacts, including increased greenhouse gas emissions and nitrate pollution (Rashid et al. 2016, Li et al. 2017b, Lourenco et al. 2019). Conservative practices such as management of understory vegetation have proven promising as alternatives for improving soil physicochemical properties and microbial diversity (Nunes et al. 2012, Araujo et al. 2013), yet such measures are often costly and require long-term application. Consequently, further development of eco-friendly, sustainable, and efficient alternative solutions for degraded land is necessary.

Introducing beneficial microorganisms to agricultural plant-soil system represents a promising strategy for improving microbial ecological functioning (Ambrosini et al. 2016), soil nutrient availability (Rashid et al. 2016), and plant productivity (Kumar and Verma 2018) and quality (Wang et al. 2019c). Such microbial inoculants most often utilize organisms, such as arbuscular mycorrhizal fungi, soil beneficial microorganisms (SBM) or plant growth-promoting bacteria (PGPB), which are usually delivered with liquid growth medium, solid peat or other organic materials to the soil or rhizosphere. These organisms can affect the soil and/or plants by direct microbial products, i.e., organic acid, hormone, extracellular polymeric substances (Costa et al. 2018), or indirectly by defending against soil-borne pathogens and inducing systemic phytochemical responses in the plant (Bashan et al. 2004, Fu et al. 2017).

With the development and increasing application of SBM (which in this chapter refers to all beneficial microorganisms such as PGPB) for improving degraded lands, microbiologists and botanists have started to decipher the mechanisms driving success or failure of such applications. For instance, it is known that specific SBM can be more effective under conditions of environmental stress (drought, salinity, low-fertility, etc.) as compared to more benign conditions (Rubin et al. 2017, Hidri et al. 2019). In addition, the resident microbial diversity

somehow determines the extent to which bacterial invaders can establish in soil, suggesting that SBM would be better able to establish themselves in soils with low resident microbial diversity (van Elsas et al. 2012, da Costa et al. 2020), such as is often the case for degraded soils. However, many factors impact the efficiency of SBM, including microbial strain properties, soil type, resident community, host plant species and phenotype, and a range of other environmental conditions. Thus, it can be a daunting task to select SBM strategies that take all relevant abiotic and biotic factors into consideration (Solano et al. 2006, Lugtenberg and Kamilova 2009, Kavamura et al. 2013).

2. Efficiency of SBM in degraded land: species selection, survival, and delivery medium level

Although the advantages of the application of microbial inoculants for soil restoration and plant improvement are widely accepted, there are still many constraints (e.g. species, survival, and delivery medium) that may compromise the efficiency of SBM, their continuous effects on soil biochemical properties, and the optimization of the effects on desired products in plants.

First of all, the efficiency and effects of SBM are highly dependent on the selection of microbial species and its functional properties. Hence, appropriate microbial species selection must be prioritized in the restoration of degraded soil-plant ecosystems. Bashan and Holguin (1998) suggested two main categories of PGPB based on their functions in plants: biocontrol-PGPB and PGPB. The former, such as *Pseudomonas* spp., act as biocontrol agents against plant pathogens in degraded land (Weller 2007). The latter comprise beneficial bacteria that restore degraded land productivity by promoting plant growth, such as *Bacillus* spp. (Kang et al. 2014) and *Azospirillum* spp. (Bashan and Holguin 1998). However, as PGPB products and biotechnology continue to develop, many strains belonging to both groups simultaneously have been introduced, and the two main categories have been divided into an increasing number of subcategories. For example, depending on the form of plant growth promotion, PGPB can be further categorized into phytostimulatory strains like *Azospirillum* sp. and *Pseudomonas* sp. (Karthikeyan et al. 2009, Walker et al. 2012, del Rosario Cappellari et al. 2013) and nutrient-enhancing strains like *Bacillus* sp. and *Azotobacter* sp. (Deng et al. 2019b, Wang et al. 2019c, Liu et al. 2020b, Wang et al. 2021b). Phytostimulatory PGPB also include a group of beneficial bacteria expressing ACC deaminase, which reduces stress-induced ethylene production in host plants under degraded or stressed conditions (Saleem et al. 2007); this group is also known as ACC deaminase-containing PGPB (Penrose and Glick 2003). Nutrient-enhancing strains include several groups that

play different roles in regulating soil nutrient availability, such as Phosphate-solubilizing bacteria (PSB) and N_2-fixing bacteria (NFB). Combining phytostimulatory and nutrient-enhancing strains can achieve multiple goals and produce significant advantages compared with single inoculation (Yu et al. 2012, Wang et al. 2021a, Wang et al. 2021b). Consequently, appropriate SBM selection depends on the roles that the beneficial microbe(s) should play in the biological restoration of degraded land.

Secondly, degraded low-fertility soil is usually a poor survival environment for introduced SBM. The introduced microbes must compete with native microbes for limited resources. Even if they outcompete specific resident taxa that share similar ecological niches in the soil, invasions are usually unsuccessful and succumb to the robust diversity in the native community (Mallon et al. 2018, Mawarda et al. 2020). Strigul and Kravchenko (2006) evaluated microbial inoculant survival in the rhizosphere and tested abiotic and biotic factors that could affect SBM survival. They found that the most important determinant of SBM survival is competition with native microbes for limiting resources. Strigul and Kravchenko (2006) also concluded that SBM were most effective in stressed and degraded soils where the development of the resident microorganisms was inhibited. Schreiter et al. (2014) found that soil type played a more important role than the inoculant itself in determining the outcome of inoculation. Hence, the fate of inoculants is likely determined by the specific environmental conditions and the growth characteristics of the introduced species (Schreiter et al. 2014). Long-term or periodic experiments are essential for unraveling the dynamics and sustainability of introduced SBM in degraded land.

At last, to improve the sustainability and efficiency of the introduced inoculant, an appropriate delivery medium is also crucial. Soil organic matter content has been recognized as a key factor limiting microbial growth, which suggests that organic amendments might help introduced microbes propagate and function in the soil (Shahzad et al. 2014, da Costa et al. 2020). Extensive study of the combined application of organic amendments and microbial inoculants has revealed that organic amendments not only improve plant growth and soil quality but also help establish stable populations of introduced microbes and enhance their effects on plants and soil (Song et al. 2015). Nevertheless, it should be noted that Rubin et al. (2017) and Strigul and Kravchenko (2006) concluded that PGPB are more effective under stressed conditions and in degraded soils. The trade-off between organic input level and inoculant efficiency should be carefully balanced. In addition, successful restoration often requires long-term bioremediation to recover soil microbial diversity (Deng et al. 2020). Consequently, the appropriate level of introduced organic materials and the application duration of microbial inoculation necessary to achieve optimal

effects on the soil and plant system should be investigated.

3. Interactions between SBM and the resident soil microbiome in degraded land

While introduced microbes may have the possibility to expand the functional potential of soils, the native soil microbial community is sensitive to exogenous disturbances (Hartmann et al. 2015, Suleiman et al. 2016). Recent work has shifted to studying how introduced microbes might impact the microbiome already resident in soils. Potential impacts of microbial inoculations have thus been expanded to examination of how such measures may reshape resident microbiome structure and function, thus representing a potential route toward improving degraded soils.

The alteration of the resident soil microbiome has been shown to be a pathway by which SBM application can lead to plant metabolome change and pathogen suppression in plant-soil systems (Badri et al. 2013, Xiong et al. 2017). However, the role to which the resident microbial diversity plays a role in this process is a matter of debate. On the one hand, microbial diversity has been shown to be a determine factor in the extent to which bacterial invaders can establish in soil (van Elsas et al. 2012, da Costa et al. 2020). On the other hand, bacterial inoculations have also been shown to induce only minor changes to the diversity of indigenous communities, with diversity not being correlated to target soil functions (Baudoin et al. 2009, Naiman et al. 2009). It is therefore important to also examine how resident community traits affect the outcome of SBM and how inoculants reshape the resident microbiome during the soil improvement process.

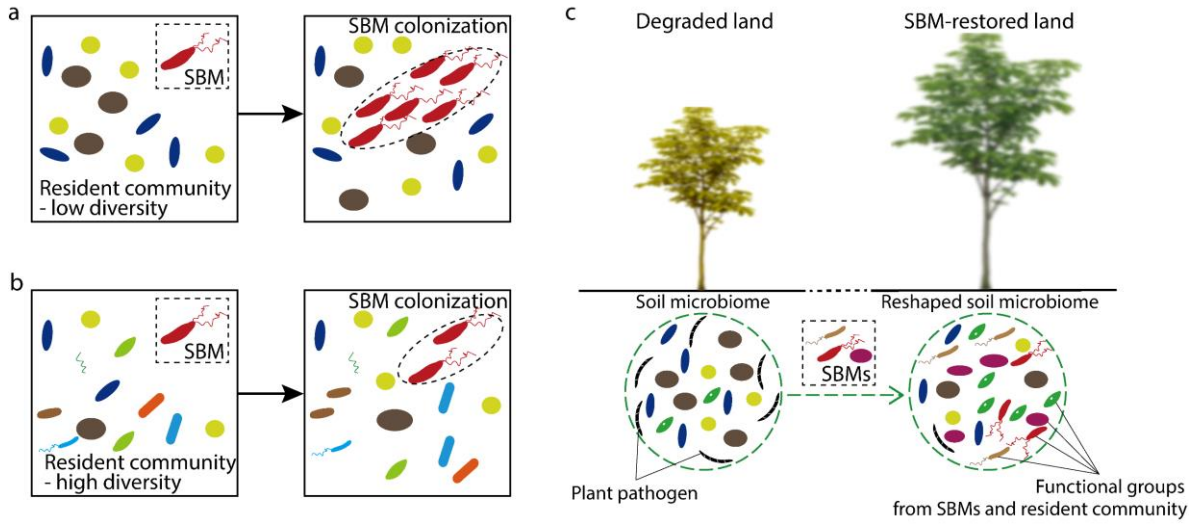


Figure 1.1 The impact of introduced SBM (soil beneficial microorganisms) on the resident soil microbial community. SBM colonization is more successful in (a) low-diversity communities than (b) high-diversity

communities. (c) SBM inoculation promotes the restoration of a degraded land ecosystem by facilitating functional microbial groups and plant pathogen resistance in the soil microbiome.

Microbial community diversity and composition can influence the degree to which introduced microorganisms can establish. For instance, van Elsas et al. (2012) reported that introduced microbes are more likely to establish in lower-diversity communities as compared to those with high microbial diversity. Given the reduced diversity often associated with degraded lands, such soils may well suited for the establishment of introduced SBM (Figure 1.1a, b). Moreover, the success of restoration also critically depends on the presence of functional microbial groups in the soil-resident community (Eviner and Hawkes 2008). Hence, as an alternative to inoculating beneficial microorganisms, a strategy for improving the efficiency of land restoration is to facilitate the populations of beneficial microbes in the native community (Figure 1.1c). For instance, Xiong et al. (2017) found that bio-fertilization resulted in significantly higher bacterial and fungal richness and phylogenetic diversity than chemical fertilization, and the changes of soil microbiome rather than the direct antagonism induced soil suppression against *Fusarium* wilt disease. Thus, the altered community structure induced by the introduction of probiotics can improve restoration efforts by facilitating the abundance of functional microbial groups in the resident community (Figure 1.1c).

With regard to the impacts of SBM on the resident community, previous studies have indicated that the responses of resident microbiome to inoculants frequently exhibit traits of resilience (Lourenco et al. 2018), resistance (Spor et al. 2020) or staying altered (Mawarda et al. 2020) to external microbial disturbances, but effects are highly dependent on the soil resources and the diversity of native microbes (Saison et al. 2006, Tardy et al. 2014). For instance, in heavy metal-polluted soil, Fernandez et al. (2012) found that AMF inoculation had no significant effects on plant growth but increased soil enzyme activity and altered microbial community composition and plant resistance under drought conditions. Similar effects were observed under semiarid conditions (Armada et al. 2014) and in soil contaminated by multiple heavy metals (Azcon et al. 2010). In summary, the introduction of microbial inoculants may cause resource competition, synergism, and antagonism effects on and within the resident microbiome (Mawarda et al. 2020). However, these authors also indicated that it remains unclear how long such impacts can persist. In addition, continuous applications of SBM may act as press disturbance and induce changes in the native microbial community (Shade et al. 2012). Consequently, the persistence and long-term impacts of

microbial inoculants in the resident community should be investigated to help understand the ecological efforts on soil functioning and plant performance in degraded lands.

4. Linking changes in soil nutrient properties to introduced SBM and reshaped soil microbiomes

Soil serves as non-renewable resource for nourishing soil microbes and animals (Scharlemann et al. 2014, Sammauria et al. 2020). The main engines of this resource are microorganisms, which improve the availability of nutrients such as phosphorus (P) and potassium (K), mobilize iron (Fe) and fix nitrogen (N) via the production of organic acids and siderophores (Rashid et al. 2016). Soil functions can be thus altered by soil microbial community composition (Don et al. 2017), particularly specific microbes such as ammonia-oxidizers (Li et al. 2018), N-fixers, and P-solubilizers (Bargaz et al. 2018). In turn, soil microbial community composition and function are determined by soil pH (Rousk et al. 2010), temperature (Zhou et al. 2016), soil organic matter and nutrient levels (Kuramae et al. 2012, Koyama et al. 2014, Martínez-García et al. 2018). Long-term chemical fertilization reduces soil nutrient availability by altering soil aggregate stability and promoting N-leaching, ammonia volatilization, and P-immobilization (Smil 2000, Gyaneshwar et al. 2002, Kaur and Reddy 2015), resulting in serious loss of soil biodiversity and degradation of soil fertility. As a sustainable soil management strategy, biofertilizers based on SBM can increase soil functionality and quality (Bhardwaj et al. 2014, Kaur and Reddy 2015), thereby increasing ecosystem services to support plant growth and productivity (Vessey 2003). Here, we summarize the effects of introduced SBM on soil restoration with respect to fertility recovery, nutrient cycling, and enzyme activities, which are proxies of microbial functionality (Doyle 1993).

Nitrogen is an essential element for nucleic acid, protein, and chlorophyll production in plants (Jnawali et al. 2015). N-transforming microorganisms play vital roles in soil and can be classified according to their roles in the transformation process (Kuypers et al. 2018). Nitrifiers and denitrifiers are responsible for nitrification and denitrification, respectively, while N-fixers perform the most important natural pathway for improving N availability in soil. Among N-fixers, comprehensive analyses have shown that N_2-fixing bacteria (NFB) can improve the soil environment under degraded conditions. In addition, soil enzymes, such as nitrogenase, urease, amidase, protease, and deaminase, are vital for the soil N cycle. Specific microbial inoculants (diazotrophs) can reduce dinitrogen to ammonium via the action of nitrogenase (De Bruijn 2015), and nitrogenase activity is a sensitive biological indicator of the impact of microbial inoculants on soil N availability (Bloch et al. 2020). For instance,

inoculation with *Azotobacter* spp. (Martinez Toledo et al. 1988), *Azospirillum* spp. (Hartmann et al. 1986), *Bacillus* spp. (Masood et al. 2020) or *Pseudomonas* spp. (Haahtela et al. 1983) improves nitrogenase activity in the soil or rhizosphere, leading to changes in soil N availability and plant N acquisition.

Phosphorus is another essential macronutrient for plants, and it is usually applied to soil as chemical fertilizer. However, immobilization rapidly makes soluble inorganic phosphate input unavailable to plants (Chen et al. 2006). Phosphate-solubilizing bacteria (PSB) release low-molecular-weight organic acids that help convert phosphate into soluble forms (Kpomblekou-a and Tabatabai 1994). Among PSB strains, *Bacillus megaterium* and *Pseudomonas fluorescens* can solubilize unavailable phosphates in degraded soil and produce a wide variety of metabolites like auxin (Kang et al. 2014, Dadrasan et al. 2015). PSB application can improve soil P availability by increasing the mobility of organic P or inducing phosphatase release (Richardson et al. 2009, Richardson and Simpson 2011). Soil phosphatases play an essential role in the P cycle by releasing inorganic phosphate from organic compounds (Sharma and Mishra 1992). *Penicillium* spp., *Rhizobium* spp., *Bacillus* spp., and *Pseudomonas* spp. have been shown to improve soil phosphatase activities (Richardson et al. 2011, Wang et al. 2019b, Wang et al. 2021b). However, phosphatase activity is inversely related to soluble P levels in the soil and thus is typically high in degraded P-deficient soil (Tadano et al. 1993). Consequently, amending the soil with nutrient-rich materials does not usually improve phosphatase activity; on the contrary, maintaining soil P at an appropriate level and introducing beneficial P-solubilizing microorganisms should generate an optimal effect.

Moreover, the nutritional constraints associated with soil degradation, such as P limitation, can also affect the NFB (Reed et al. 2011, Divito and Sadras 2014). Hence, a comprehensive approach to improving soil nutrient properties is necessary to mitigate such potential limitations. The combined application of different beneficial strains with different traits provides significant advantages compared with the use of a single microbial inoculant (Juge et al. 2012, Yu et al. 2012, Hungria et al. 2013). For instance, detailed studies have shown that combined inoculation with PSB and NFB has great potential to enhance plant growth and soil nutrients under degraded conditions (Wang et al. 2019c, Wang et al. 2021b). The advantages of co-inoculation are the result of synergistic interactions that stimulate physical or biochemical activities and simultaneously improve microbial viability (Yu et al. 2012), thus enhancing the benefits to the soil and host plant through the production of enzymes and organic acids. The potential synergetic effects of mixed inoculants such as PSB and NFB on soil nutrient availability under low-fertility conditions warrant further exploration.

SBM introduction can indirectly alter soil fertility, functionality and biogeochemical processes by impacting resident soil microbiome functioning. For instance, the introduced microbes could outcompete specific taxa that share similar ecological niches in the soil (Mallon et al. 2018, Mawarda et al. 2020). SBM inoculation in degraded soil can also produce antagonistic effects on resident pathogens via antibiotic production (Dukare et al. 2019). However, these antibiotics might also influence the populations of other microbial taxa in the soil and lead to changes in community functioning. In addition, biofertilizers containing SBM can also induce synergistic effects on beneficial microbial groups such as *Pseudomonas* spp. and *Bacillus* spp., thus explaining the changes of soil nutrient status (Xiong et al. 2017, Wang et al. 2021a). Consequently, future studies should not ignore changes in the soil microbiome as a driver of the dynamics of soil functioning, such as soil enzyme activities and soil nutrient cycling (Ma et al. 2018).

5. From belowground to aboveground: plant cultivation in degraded land

Plants grown in degraded land frequently suffer from abiotic and biotic stresses that constrain primary growth but stimulate the accumulation of secondary metabolites. Plant metabolites exhibit a diverse range of biological activities, such as mitigation of environmental stress and plant defense against herbivores and soil-borne pathogens, which can enhance plant performance in degraded land. The symbiotic relationship between plants and soil microorganisms increases the ecological adaptability of plants to environmental constraints (Selosse and Le Tacon 1998, Brundrett 2002). Thus, introducing beneficial microorganisms can help plants alleviate stress by altering plant physiological responses such as metabolite accumulation. For instance, phytostimulatory strains like *Azospirillum* sp. and *Pseudomonas* sp. participate in synergistic metabolic activities with the host plant to increase the biosynthesis of certain secondary metabolites (Karthikeyan et al. 2009, Walker et al. 2012, del Rosario Cappellari et al. 2013). Other strains, like *Bacillus* sp. and *Azotobacter* sp., can be used to improve soil nutrient properties and the plant's internal nutrient stoichiometry, which are vital in modulating plant metabolism and metabolite production (Deng et al. 2019b, Wang et al. 2019c, Liu et al. 2020b, Wang et al. 2021b). Moreover, SBM promote beneficial microbial groups in the rhizosphere to enhance plant performance under stress (Xiong et al. 2017).

Inoculation changes the plant metabolome both indirectly via differences in soil nutrient availability and directly via the induction of systemic resistance. However, few studies have examined the contribution of the inoculant-

reshaped soil microbiome. Shifts in the soil microbiome are associated with plant primary growth in various ways, most notably by influencing the accumulation of plant secondary metabolites, which are usually related to plant primary growth parameters such as biomass production (Hol 2011). Manipulation of the soil microbiome by soil inoculation was recently shown to influence the concentrations of pyrrolizidine alkaloids (PAs) in *Jacobaea vulgaris* plants (Wang et al. 2019a, Huberty et al. 2020), and in another study, changes in the soil microbiome were induced by plant cultivation (Kos et al. 2015). These observations suggest that altering the soil microbiome might reprogram plant physiology as well as plant defense (Li et al. 2019, Pineda et al. 2020). Hence, it is important to understand the underlying mechanisms by which SBM and associated soil biota affect the plant metabolome.

Although efforts have begun to identify the key soil microbial groups associated with the accumulation of secondary metabolites in plants (Badri et al. 2013, Pineda et al. 2020, Zhang et al. 2020), elucidating the precise mechanisms by which inoculated organisms elicit a specific plant phenotype remains challenging due to the multiple players and complex interactions involved. The use of inappropriate analysis methods when integrating microbiome data, plant and soil variables, and other factors in the whole system could generate unreliable and one-sided results (B. Sohn and Li 2018, Leite and Kuramae 2020). In particular, simple correlation analysis may lead to false significant relationships between the soil microbiome and plant performance because of the compositional nature of soil microbiome data (Gloor et al. 2017). The reliability of this method and the conclusions derived from it are questionable, but unfortunately, many studies continue to use simple correlation analysis to identify relationships between the soil microbiome and plant metabolome (Badri et al. 2013, Pineda et al. 2020, Zhang et al. 2020). A recent study (Leite and Kuramae 2020) supported the superior performance of model-based approaches in characterizing the soil microbiome and the impacts of microbiota on the ecosystem. This gap in methodology for evaluating the relationship between the soil microbiome and plant metabolome and other plant-soil-microbe interactions is an important direction for future research.

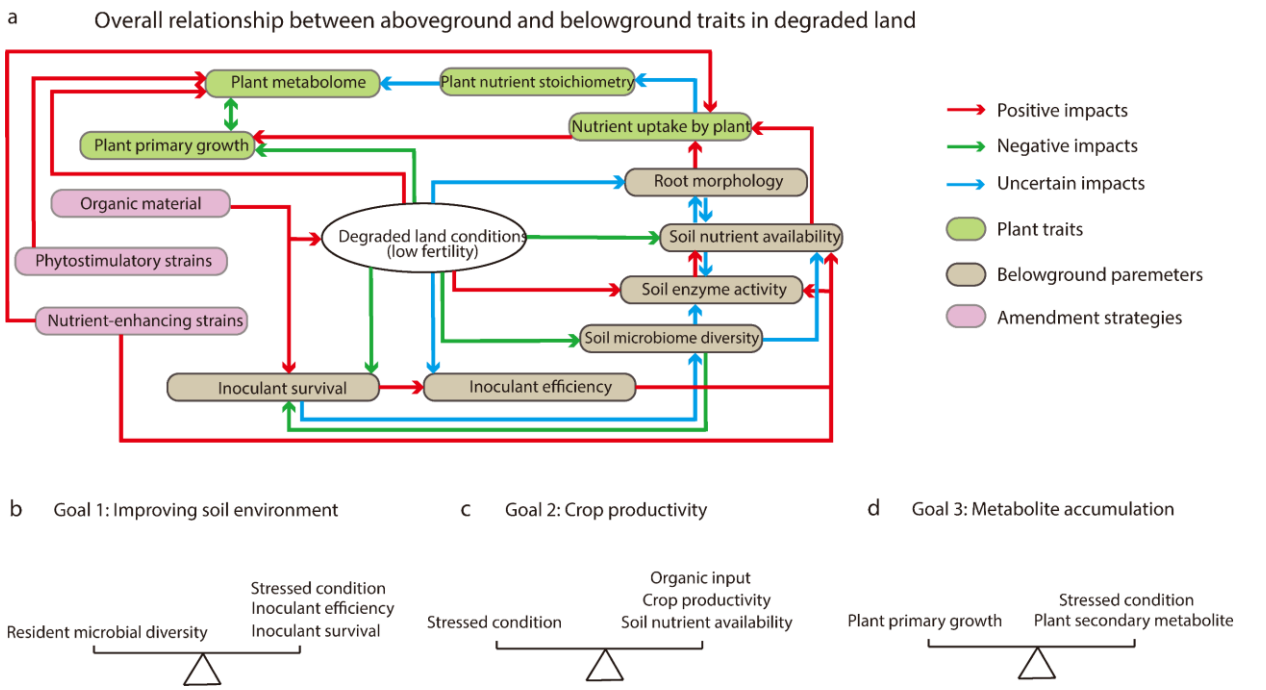


Figure 1.2 The network of relationships in degraded land ecosystems in response to different amendment strategies. (a) Overall relationship between aboveground and belowground traits; uncertain impact (blue arrow) means the impact could be positive or negative, depending on other variables in the ecosystem. To simplify the network flow for different research and farming goals, panels b, c, and d explain the trade-offs that should be considered to improve soil fertility, crop productivity, and secondary metabolite accumulation, respectively.

It appears that increasing the production of plant secondary metabolites may be advantageous for improving plant adaptability to degraded land conditions and their downstream application value, such as for use as medicinal plants. However, the introduction of SBM usually improves plant primary growth such as biomass accumulation, which may limit the accumulation of secondary metabolites (Figure 1.2a). In other words, enriching the soil environment via amendment strategies may actually result in lower plant metabolite accumulation than observed for unimproved degraded conditions, which raises the question of how best to introduce SBM in degraded land in order to achieve higher concentrations of target metabolites in the host plants without also enhancing primary growth. Furthermore, the addition of materials with rich nutrients may reduce the effects of microbial inoculants since SBM may be more effective under stressed conditions (Rubin et al. 2017) (Figure 1.2a). Consequently, the relationship between stressed conditions, organic input, inoculant survival, and plant productivity should be balanced to minimize economic input while optimizing the effect of introduced beneficial microbes on the desired outcome, both in terms of land restoration as well as production value. Figure 1.2 illustrates the relationship

network between belowground and aboveground traits under low-fertility conditions and how these trade-offs should be considered to select appropriate and balanced amendment strategies for different ecological goals (Fig 2b, c, d).

6. Thesis Outline

The negative side effects of chemical input prompts new more environmentally friendly methods of improving degraded land management, such as the utilization of SBM. Although introducing SBM to degraded land represents a promising strategy, the effects are highly dependent on the SBM species used, inoculation times and period, fertilizer level, and resident microbial community characteristics. All these factors impact the effectiveness of such treatments, making it important to study the individual and interactive effects that have during SBM applications. Furthermore, the introduced SBM can impact the resident microbial community composition and succession during the restoration progress, but less is known about the subsequent influences of soil microbiome changes on plant performance and soil functioning. Newly-developed biotechnologies and methods for obtaining and analyzing microbiome data have provided the opportunities to decipher the underlying mechanisms of plant-soil-microbe interactions and help select appropriate SBM for a given degraded land condition or a desired plant production.

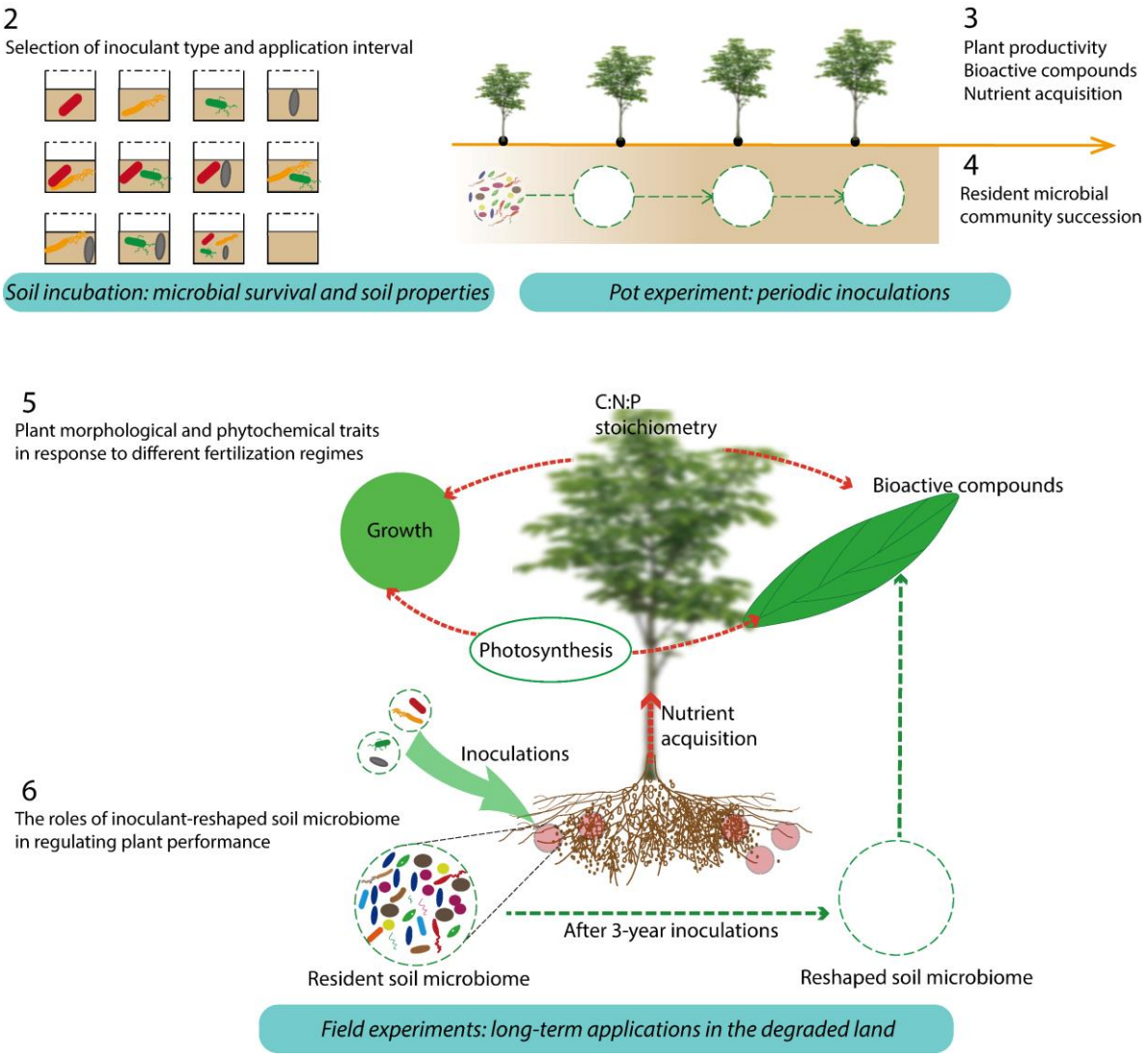


Figure 1.3 Schematic overview of the chapters presented in this thesis.

The main aims of this thesis are:

(1) to select appropriate SBM inoculants, their combinations, and application period based on microbial growth dynamics in the degraded soil and their effects on soil biochemical properties.

(2) to evaluate the impacts of periodic applications of selected SBM on plant growth, biomass distribution, and bioactive compounds of a native medicinal plant, *Cyclocarya paliurus*.

(3) to reveal the succession traits of the resident microbial community under periodic SBM applications and reveal the underlying biotic and abiotic factors.

(4) to investigate the impact of bio-fertilizer forms (organic fertilizer with/without SBM) and levels (low, medium, high) in affecting soil nutrient properties, plant morphological and physiological traits in degraded field

conditions.

(5) to connect the introduced SBM inoculants and reshaped soil microbial community to improved plant growth performance and medicinal value under pot and field conditions.

To address these questions, I applied a combination of experimental approaches, including controlled experiments in the lab, a 1-year pot experiment and a 3-year field experiment under natural conditions (Figure 1.3).

In **chapter 2**, we assessed the survival ability of different SBM inoculants (single or mixed inoculants from four commercial strains) in degraded soil and evaluated their effects on soil nutrient characteristics using a 60-day soil incubation experiment under controlled conditions. Our results demonstrated that co-inoculations with phosphate-solubilizing bacteria (PSB: *Bacillus megaterium* and *Pseudomonas fluorescens*) and N_2-fixing bacteria (NFB: *Azospirillum brasilense* and *Azotobacter chroococcum*) performed better than single-strain inoculants in stimulating soil nutrient contents. The inoculation should be conducted at intervals of 30-45 days to maintain microbial populations in the degraded soil.

In **chapter 3**, we evaluated plant growth, biomass distribution, and metabolic profiles of *Cyclocarya paliurus* in response to different inoculant types (PSB, NFB, PSB+NFB) in a pot experiment. In addition, we investigated the relationship between C: N: P stoichiometry and bioactive compounds in *C. paliurus* leaves. Our results suggested that SBM inoculations improved plant growth and metabolites yield by altering the nutrient availability in soil and C: N: P stoichiometry in the leaves, but the three inoculant types showed different patterns with inoculation with four strains together showing better performance than single bacterial additions.

In **chapter 4**, we examined the comprehensive responses of plant dynamic growth, soil functioning, and the resident microbial community to periodic inoculations (four times at 45-day intervals) of PSB and NFB alone or in combination through a growing season. Our results showed that co-inoculation stimulated plant growth and improved soil nutrient levels better compared to single-strain inoculations. The periodic inoculations impacted the succession course of resident bacterial communities in bulk soil, mainly driven by changes in soil pH and nitrate, resulting in the development of three main community clusters throughout the investigation. The different microbial inoculants showed distinct impacts on resident microbiome succession, with affected communities ultimately showing resilience in community structure.

In **chapter 5**, we combined controlled laboratory experiments with field trials to investigate the effects of co-inoculation with phyto-stimulatory strains (*Azospirillum brasilense* and *Pseudomonas fluorescens*) and nutrient-

enhancing strains (*Bacillus megaterium* and *Azotobacter chroococcum*). SBM inoculants were applied with organic fertilizer at different fertilizer levels, and we tracked effects on soil nutrient availability as well as *C. paliurus* 3D morphological traits, photosynthesis, growth and bioactive compounds. Our results showed that bioactive compounds in *C. paliurus* leaves can be affected by internal nutrient balance, which is associated with altered root system morphology that was modulated by bacterial inoculation. Both fertilizer type and level should be considered when tailoring management regimes to achieve desired cultivation objectives.

In **chapter 6**, we applied generalized joint attribute model (GJAM) analysis to examine the impacts of bio-fertilizer level and probiotic consortia on soil microbiome assembly, plant nutrient stoichiometry and plant metabolic content over three successive years under field conditions. Crucially, we investigated the tripartite relationship between the resident soil microbiome, introduced SBM inoculants, and plant performance. The results showed that probiotic consortia can modulate plant metabolites by conditioning the soil microbiome and plant nutrient balance. Specific microbial taxa could be identified as indicators for selecting appropriate fertilization regimes and improving plant metabolic performance.

In **chapter 7**, I summarize the results presented in this thesis and discuss them in the broader context of plant–soil–SBM interactions. Furthermore, I provide suggestions and identify current knowledge gaps as targets for future research.

Chapter 2 Screening the appropriate inoculants for degraded soil amendment based on microbial species, combinations, survivals and effects on soil properties

Adapted from: Wang Z, Chen Z, Fu X. Integrated effects of co-Inoculation with phosphate-solubilizing bacteria and N_2-Fixing bacteria on microbial population and soil amendment under C deficiency[J]. International Journal of Environmental Research and Public Health, 2019, 16(13): 2442.

Abstract

The inoculation of beneficial microorganisms is a promising soil amendment strategy to improve plant growth and soil properties. However, the effects of co-inoculation with phosphate-solubilizing bacteria (PSB) and N_2-fixing bacteria (NFB) in typical C-deficient soil remain unclear. Furthermore, the dynamic microbial populations and their relationships with soil functioning should be revealed. Based on a controlled experiment and a pot experiment, we examined the effects of PSB (M: *Bacillus megaterium* and F: *Pseudomonas fluorescens*), NFB (C: *Azotobacter chroococcum* and B: *Azospirillum brasilence*), and combined PSB and NFB treatments on C, N, P availability, and enzyme activities in sterilized soil, as well as the growth of *Cyclocarya Paliurus* seedlings grow in unsterilized soil. During a 60-day culture, prominent increases in soil inorganic N and available P contents were detected after bacteria additions. Three patterns were observed for different additions according to the dynamic bacterial growth: early unimodal, unimodal, and bimodal. Synergistic effects between NFB and PSB were obvious, co-inoculations with NFB enhanced the accumulation of available P. However, decreases in soil available P and N were observed on the 60th day, which might be induced by the decreases in bacterial quantities under C deficiency. Besides, co-inoculations with PSB and NFB resulted in greater performance in plant growth promotion. Aimed at amending soil with a C supply shortage, combined PSB and NFB treatments are more appropriate for practical fertilization at intervals of 30–45 days. The results demonstrate that co-inoculations could have synergistic interactions during culture and application, which may help with understanding the possible mechanism of soil amendment driven by microorganisms under C deficiency, thereby providing an alternative option for amending such soil.

1. Introduction

In Southern China, plantation areas are mostly assigned to poor sites with yellowish-brown clay soil in the subtropical mountainous areas, as part of the Grain for Green Project (GTGP). These regions are perceived to be infertile due to low levels of organic C and nutrient contents (Fazhu et al. 2015, Huang et al. 2017). Many studies have been emphasizing efficient soil amendment strategies to improve the poor status of such soils (Fazhu et al. 2015, Tang et al. 2016). However, the most common strategy, chemical fertilization, has produced harmful effects in the soil and the environment (Bhardwaj et al. 2014). Due to leaching and immobilization (Smil 2000), few nutrients, such as N and P, in the soil are available for plant uptake even after long-term chemical fertilizer treatment (Gyaneshwar et al. 2002, Adesemoye and Kloepper 2009, Kaur and Reddy 2015). These problems are

now compelling researchers to find more sustainable and advanced techniques to remediate the soil (Megali et al. 2014, Yin et al. 2018). Of the recommended strategies, the use of bio-fertilizer has proven to be an efficient and eco-friendly management practice in improving soil fertility and crop growth (Liu et al. 2014).

A bio-fertilizer is a substance containing living beneficial microorganisms that can colonize the rhizosphere and stimulate plant growth by increasing the supply of available nutrients to plants when applied to the soil (Vessey 2003). Soil N and P are known to be two of the most essential nutrients for plant growth and development worldwide. As tested soils in South China are seriously lacking in available N and P, the fixation of N and solubilization of P driven by N_2-fixing bacteria (NFB) and phosphate-solubilizing bacteria (PSB) are of central importance. NFB have the ability to convert inert N_2 into ammonium and thereby protect nitrogen from being lost through volatilization and leaching (Yevdokimov et al. 2008). PSB can convert insoluble phosphates into a bio-available form through solubilization and mineralization (Behera et al. 2017).

Soil C plays a crucial role in the application of bio-fertilizer, which is one of three soil components crucial for its physical and biochemical properties, and the degradation of organic matter is closely related to soil microbial activity (Debska et al. 2016, Yilmaz and Sonmez 2017). Several studies have reported different findings regarding the effects of bio-fertilization on soil C content (Huang et al. 2013). In turn, soil microbial populations and enzyme activities are related to organic C input (straw, compost, and manure), which could reduce the negative effects of the severe environment on microorganisms (Gunasekara and Xing 2003). Many reports have highlighted the effects of microorganisms input on soil nutrient content, plant growth, and disease resistance, as well as the importance of soil C when applying microorganisms to the soil (Yu et al. 2012, Megali et al. 2014, Yilmaz and Sonmez 2017). However, most approaches were conducted using a single bacteria strain, which may partially account for the recorded inconsistencies in the field (Hameeda et al. 2008, Valetti et al. 2018). Hence, less is known about the effects of co-inoculation with PSB and NFB on soil properties. The soil amending mechanism and interactions between NFB and PSB under C-deficiency remain to be determined.

The effects of bio-fertilizer evaluated in other areas are often limited by different factors, such as incubation time, inoculation types, limited C resources and survival of microbes (Hu et al. 2017). On the other side, soil native microbes could influence the effects of bio-fertilizer on plant growth. Therefore, the characteristics of the typical soil in subtropical mountainous plantation areas, the time-effectiveness of inoculants, and the selection of the appropriate beneficial microorganism combination for fertilization should be investigated. The aims of this study

were to determine the adaptive bacterial isolates or combinations and their application period, specific attention was focused on the soil amendment mechanism and interaction between NFB and PSB under C-deficiency. Basically, a lab experiment was conducted to investigate, (1) whether these microorganisms could survive and multiplicate under limited C resources, (2) their efficiency in improving the main soil nutrient contents (N and P) in yellowish-brown clay soil under sterilized conditions. As supplementary, a pot experiment was conducted under non-sterilized soil conditions, to verify the effects of these strains accompanied by the native microbes, on plant growth and biomass accumulation. These results could interpret the mechanism of action and interaction between bacteria strains and soil with different incubation time under C-deficient conditions, as well as provide supports for the application of bio-bacterial fertilizer in such soils.

2. Materials and Methods

2.1. Soil Properties and Pretreatment

Natural soil was sampled from the top layer (0–20 cm) at a *Cyclocarya Paliurus* plantation (a typical medicinal plant in subtropical regions in China) in July 2016, which was located in Baima Nanjing (31°35′ N, 119°10′ E), China. Samples were collected from five plots (1 × 1 m) in an "S" pattern in 4-year-old *C. Paliurus* plantation fields (about 120 × 40 m, at a planting density of 2 × 2 m) and were mixed thoroughly to form a composite sample. After removing the plant material, stones, and other debris, the collected soil was divided into two parts, one was sieved (2 mm) and kept at 4 ℃ prior to use in the lab experiment, the other one was used for pot experiment.

The above soil is the representative soil type in subtropical regions in China, which was classified as yellowish-brown clay soil with a heavy texture, pH of 6.5, bulk density of 1.6 $g \cdot cm^{-3}$, total C of 4.1 $g \cdot kg^{-1}$, total N of 0.79 $g \cdot kg^{-1}$, total P of 0.30 $g \cdot kg^{-1}$, total of K 0.10 $g \cdot kg^{-1}$, NH_4^+–N of 10.94 $mg \cdot kg^{-1}$, NO_3^-–N of 2.68 $mg \cdot kg^{-1}$, and available P of 1.03 $mg \cdot kg^{-1}$.

2.2. Microorganisms

In this study, we used four microorganisms, including phosphate solubilizing bacteria (PSB, viz., M: *Bacillus megaterium* and F: *Pseudomonas fluorescens*) and N_2-fixing bacteria (NFB, viz., C: *Azotobacter chroococcum* and B: *Azospirillum brasilence*). The above bacteria have been documented as having the ability to improve soil

nutrients, such as N and P (Venieraki et al. 2011, Kumar et al. 2012, Mengual et al. 2014, Ortiz et al. 2015, Wyciszkiewicz et al. 2016). Prior to use, the inocula were prepared by incubating bacteria strains in a lysogeny-broth medium (LB medium, pH: 7.0, comprised of 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter). At the mid-exponential growth phase, the strains were diluted using sterile distilled water to a final concentration of 1 × 10^8 colony forming units (CFU)·mL^{-1}. None of these strains have shown antagonistic effects against one another (Mittal et al. 2008, Yu et al. 2012, Dadrasan et al. 2015).

2.3. Experimental Design

In the lab experiment, the soils were incubated with 12 additions (treatments) of the 4 bacteria, containing 4 treatments with a single bacteria addition (SBA), 7 treatments with a mixed bacteria addition (MBA), and 1 control with no bacteria addition (Table 2.1). Each treatment was replicated 4 times, and the bacteria were added to the soil, which was autoclaved enough times to eliminate other microbes. Thereafter, 300 g of sterilized soil supplemented with bacteria was placed into a cylindrical tissue-culture box (diameter (D) × height (H): 8.5 × 8.4 cm, breathable and waterproof), and the box was incubated in a bio-clean incubator at 28 °C under darkness conditions for 60 days. During incubation, the soil moisture was held at 60% of the water holding capacity with sterile water.

The pot experiment was conducted based on the lab experiment results, with three types of bacteria combination (PSB: M, MF; NFB: C, CB; PSB+NFB: MFCB). An important medicinal species (*C. Paliurus,* 2-year-old seedlings) native to China's subtropical mountainous area, was grown in the same soil as we used in this study without sterilization. From April, four times of bio-fertilizations were conducted every 45 days according to bacterial growth results.

Table 2.1 Soil with 12 additions with different bacteria combinations (mL).

Inoculants Type	**Treatment**	**M: *Bacillus megaterium***	**F: *Pseudomonas fluorescens***	**C: *Azotobacter chroococcum***	**B: *Azospirillum brasilence***
SBA	M (PSB)	5	0	0	0
	F (PSB)	0	5	0	0
	C (NFB)	0	0	5	0
	B (NFB)	0	0	0	5

	MF	2.5	2.5	0	0
	MC	2.5	0	2.5	0
	MB	2.5	0	0	2.5
MBA	FC	0	2.5	2.5	0
	FB	0	2.5	0	2.5
	CB	0	0	2.5	2.5
	MFCB	1.25	1.25	1.25	1.25
Control	Control	0	0	0	0

SBA: single bacteria addition; MBA: mixed bacteria addition; PSB: phosphate solubilizing bacteria; NFB: N_2-fixing bacteria

2.4. Sampling and Analytical Methods

The soils in the lab experiment were vertically sampled on the 5th, 10th, 15th, 20th, 30th, 45th, and 60th days of incubation (Figure 2.1) to estimate the bacterial quantity (BQ) using the plate count serial dilution method (Sanders 2012). Similarly, soil samples from each box on the 0th, 30th, and 60th day of incubation were collected and stored at 4 °C for measurement of the soil properties. Total C (TC) and total N (TN) were evaluated using an elemental analyzer (vario MAX CN, Elementar, Hanau, Germany), where the concentration of inorganic N (IN, including NH_4^+–N and NO_3^-–N) was extracted with a 2 M KCl solution, and then measured by colorimetry on an AutoAnalyser III (SEAL Analytical, Berlin, Germany). Soil available P (SAP) was determined using the molybdenum-blue method (Olsen 1954). Acid phosphatases (AcPase) activity was assessed using the method described by Tabatabai and Bremner (Tabatabai and Bremner 1969). Each experiment was conducted in three replicates for measurements of the BQ and soil properties.

For the measurement of plant growth in the pot experiment, the whole plants were sampled in late September to assess the biomass accumulations (including stem, root, and leaf). Seedling heights were measured by the difference of initial (April) and final height (late September).

Figure 2.1: Abridged general view of soil sampling. Five random vertical sa5mpling holes (diameter: 8 mm; depth: 60 mm) were implemented for lessening the disturbance of sampling to microbes.

2.5. Statistical Analysis

The Shapiro–Wilk test and Levene's test were used for testing the normal distribution of the data and homogeneity of the variances, respectively. Mixed linear models were used to assess the effects of the inoculant, incubation time, and their interactions (as fixed effects), as well as the block as a random effect on the soil's biochemical properties. Where there were significant effects ($p < 0.05$), the Duncan's multiple range test was applied to determine the differences between the individual treatment means. Tamhane's T_2 was used to test for differences amongst treatments when variances of the tested data were not equal. Data are expressed as means ± standard deviation (SD). All statistical analyses were considered significantly at $p<0.05$. The pairwise relationships of BQ and P-related indexes were elucidated using linear regression based on Spearman's correlation analysis. All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Dynamic growth of Bacteria in Incubation Soil

Generally, the bacterial quantities (BQ) in all the treatments significantly increased with prolonged incubation, whereas the maxima were obviously different between the mixed bacteria addition (MBA) and the single bacteria addition (SBA). The maximum values of the BQ for MBA ranged from 18.3×10^6 CFU·g^{-1} in MB to 43.3×10^6 CFU·g^{-1} in MFCB, whereas for SBA, they ranged from 8.3 CFU·g^{-1} in M to 17.3×10^6 CFU·g^{-1} in C (Table 2.2). Based on the dynamic changes in bacterial growth, three patterns were observed for the different additions (Figure 2.2). The

peaking of the BQ for SBA occurred at different times from that in MBA; the quantity in SBA peaked at the 15–20th day and the peaks in most of the MBA (MC, CB, MB, FC) occurred at the 30th day, whereas some (FB and MF) presented bimodal peaks at the beginning and midterms of incubation (Figure 2.2).

Quantities of the two functional bacteria varied with incubation length. Quantities of the phosphate-solubilizing bacteria (M and F) appeared to decline in the last 30 days, while N_2-fixing bacteria (C and B) increased (Table 2.2 and Figure 2.2a). On the 60th day, 17.3 × 10^6 and 12.7 × 10^6 CFU·g^{-1} in C and B, respectively, were significantly higher than the 1.9 × 10^6 and 3.6 10^6 CFU·g^{-1} in M and F, respectively ($p < 0.05$). Overall, the single bacteria grew rapidly without competing pressure compared to other combinations, reaching their peak quickly and with a low maximum quantity. Conversely, the competition of mixed bacteria retarded the peaking time but increased the maximum.

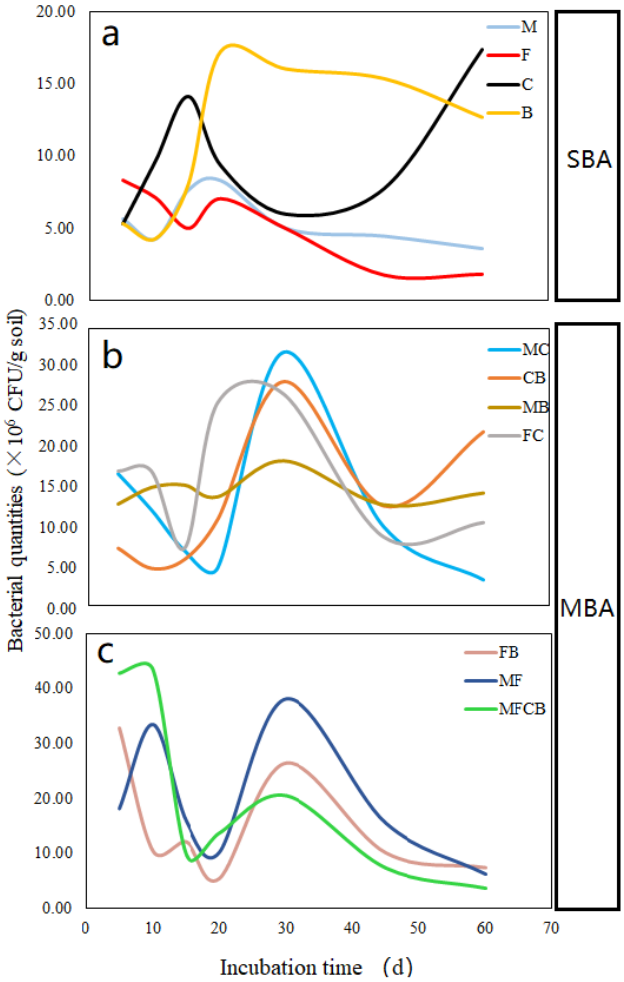


Figure 2.2: Three dynamic growth patterns of different inoculants during the 60-day incubation. (**a**) pattern 1, a single peak observed at 10–20 d by single bacterium addition. (**b**) pattern 2, a single peak observed at 30–40 d by mixed bacteria addition. (**c**) pattern 3, bimodal observed at different time by mixed bacteria additions

Table 2.2. Changing patterns of bacterial quantities (×10^6 CFU·g^{-1} soil) for bacterium addition with different incubation duration (means ± standard deviation).

Treatment	Incubation Duration (d)							
code	0	5	10	15	20	30	45	60
MFCB	0.167 ± 0	42.7 ± 5.3Aa	43.3 ± 4.7Aa	9.7 ± 1.5Dc	13.6 ± 3Cbc	20 ± 0.6Bd	7.3 ± 1DEbcde	3.7 ± 1Fefg
MF	0.167 ± 0	18C ± 0.3c	33.3 ± 1.9Bb	15.67 ± 1.3Da	10 ± 1.2Ecd	38 ± 1.2Aa	15.33 ± D1a	6.1 ± 1Fef
FC	0.167 ± 0	17 ± 6Bc	17 ± 5.5Bc	7.2 ± 1DEde	25.6 ± 3.8Aa	26.6 ± 3.2Ac	8.7 ± 2.8CDbc	10.5 ± 2Ccd
MB	0.167 ± 0	12.9 ± 2BCc	15 ± 1.6ABc	15.3 ± 2ABa	13.8 ± 1Bc	18.3 ± 2Af	12.8 ± 2.7BCab	14.3 ± 0.3Bc
FB	0.167 ± 0	33 ± 4.3Ab	10.3 ± 4.1Ccde	12 ± 2Cb	5.3 ± 1DEefg	26.3 ± 3.5Bc	10 ± 3.2Cabc	7.3 ± 2Dde
CB	0.167 ± 0	7.3 ± 2Dd	4.8 ± 2.3DEFde	5.9 ± 1.8DEde	11 ± 1Ccd	28.3 ± 5.7Ac	12.7 ± 5Cab	22 ± 5.3Ba
MC	0.167 ± 0	16.7 ± 5Bc	12.1 ± 0.2Ccd	7 ± 1DEde	4.8 ± 1.8EFfg	32 ± 2.6Ab	10 ± 3.5CDabc	3.3 ± 1FGefg
B	0.167 ± 0	5.3 ± 0.6Ed	4.3 ± 2.2EFe	8.0 ± 1Dde	17.2 ± 3.5Ab	16 ± 0.8ABe	15.3 ± 4ABCa	12.7 ± 3Cc
C	0.167 ± 0	5.3 ± 3.2DEFd	9.7 ± 1.5Ccde	14.0 ± 0.5Bc	9.3 ± 3Cde	6.0 ± 1DEf	7.7 ± 1CDbcd	17.3 ± 2Ab
F	0.167 ± 0	8.3 ± 1.5Ad	7.1 ± 2.5ABde	5.0 ± 0.4BCe	7.1 ± 3ABdefg	5.0 ± 0.8BCf	1.8 ± 0.8De	1.9 ± 0.7Dg
M	0.167 ± 0	5.6 ± 1.5BCd	4.2 ± 1.6CDEe	7.7 ± 1.5ABcd	8.3 ± 3Adef	5.0 ± 0.6CDf	4.5 ± 0CDEcde	3.6 ± 1DEefg

* M, F, C, B: single inoculation with M (*Bacillus megaterium*) or F: (*Pseudomonas fluorescens*) or C (*Azotobacter chroococcum*) or B (*Azospirillum brasilence*). MC, CB, FB, MB, FC, MF: dual inoculation with M and C, C and B, F and B, M and B, F and C, M and F. MFCB: mixed inoculation with four strains. Different capital letters denote significant differences among incubation durations at $p < 0.05$, different lowercase letters denote significant differences among treatments at $p < 0.05$ on the same incubation duration.

3.2. Inoculants, Incubation Time, and Their Interactions on Soil Characteristics

Based on the statistical analysis results, the effects of the inoculants, incubation duration, and their interactions on soil TC, TN, IN, available P, and P-related enzyme activities are presented in Table 2.3. Over a 60-day incubation, we found that TC and TN showed significant responses to incubation time, whereas no significant effects of inoculant addition on TC and TN were detected (p = 0.07 and 0.06, Table 2.3). IN (NH_4^+–N + NO_3^-–N), available P, and P-related enzyme activities of the incubation soil were significantly affected by inoculant additions and incubation duration (p < 0.01). Interactions of the inoculants and incubation duration were significant for all measured parameters (p < 0.05, Table 2.3).

Given the significant effects of incubation time on these indexes, pairwise comparisons of the indexes between 30 days and 60 days were analyzed for all additions (Table 2.4). Impacts of incubation duration on SAP existed in each inoculant except CK. However, the effects of incubation duration on other soil parameters varied with different inoculants.

Table 2.3. The linear mixed model for the effects of inoculants, incubation time, and their interactions on soil characteristics.

Variables	Inoculants		Incubation Duration		Inoculants × Incubation Duration	
	F-test	*Sig.*	F-test	*Sig.*	F-test	*Sig.*
TC	1.82	nd	54.48	**	2.03	*
TN	1.86	nd	43.11	**	2.93	**
NH_4^+-N	10.90	**	24.57	**	3.11	**
NO_3^--N	20.27	**	48.50	**	5.87	**
SAP	26.58	**	2162.43	**	21.40	**
AcPase	24.42	**	67.18	**	21.34	**
IN	19.18	**	75.34	**	7.65	**

TC: total carbon; TN: total nitrogen; SAP: soil available phosphorus; IN: inorganic nitrogen; *Sig*: significance, * indicates p values < 0.05, ** indicates p values < 0.01, nd indicates significance not dectected.

Table 2.4. Pairwise comparisons' results of soil indexes between 30d and 60d incubation for all additions.

Inoculants		TC	TN	NH_4^+-N	NO_3^--N	SAP	AcPase	IN
		30d-60d	30d-60d	30d-60d	30d-60d	30d-60d	30d-60d	30d-60d
SBA	M	**	**	**		**	nd	**
	F	nd	*	*	*	**	*	**
	C	*	nd	nd	nd	**	nd	nd
	B	nd	nd	**	nd	**	nd	**
MBA	MF	**	**	**	*	**	**	**
	MC	**	*	nd	**	**	**	**
	MB	*	*	nd	**	**	*	nd
	FC	*	**	nd	**	**	**	nd
	FB	*	**	*	**	**	**	**
	CB	nd	nd	*	nd	**	*	**
	MFCB	nd	nd	*	nd	**	**	**
Control	CK	nd	nd	nd	nd	nd	nd	nd

3.3. C and N Contents of the Incubation Soil

As shown in Table 2.5, the results show the significant effects of bacteria additions on TC and TN after a 30-day incubation ($p < 0.05$). Prominent increases in TN content were found in MF, FB, and FC at 30 days compared to the control (0.7 $g \cdot kg^{-1}$). However, variations in TC and TN contents among different bacteria additions were not significant at 60 days, but obvious reductions occurred in both SBA and MBA at 60 days compared to 30 days (Table 2.4; Table 2.5; $p < 0.05$). For instance, significant decreases in TC in treatments M, C, and MC were detected at 60 days compared to 30 days (decreased by 12.1%, 7.4%, and 16.1%, respectively), whereas obvious reductions of N in MF, MC, MB, FC, and FB were recorded (Table 2.5).

Differences in IN contents were observed after additions of various bacteria and two incubation durations (Table 2.4; Table 2.5). A significant increase in soil IN content was detected in the first 30 days after bacteria addition (Table 2.5, $p < 0.05$). However, in contrast to the 30 days, the IN contents of most treatments at 60 days were lowered but were still significantly higher than in the control (Table 2.4, $p < 0.01$). Statistically, no remarkable

changes were detected under conditions of inoculation with NFB alone (except B at 30 days) compared to the control.

Table 2.5. Incubation soil TC, TN and IN contents after beneficial bacteria addition.

Inoculants		TC (g·kg^{-1})		TN (g·kg^{-1})		IN (mg·kg^{-1})	
		30 d	60 d	30 d	60 d	30 d	60 d
SBA	M	4.6 ± 0.2a	4.0 ± 0.3a *	0.9 ± 0.1a	0.7 ± 0.1a *	34.8 ± 1.6b	19.6 ± 2.4cd *
	F	4.1 ± 0.2a-d	3.9 ± 0.1a	0.9 ± 0.1ab	0.7 ± 0.1a *	34.3 ± 2.5bc	23.9 ± 6.6abc *
	C	4.3 ± 0.2a-d	4.0 ± 0.2a *	0.7 ± 0.1c	0.7 ± 0.1a	10.7 ± 2.8f	9.7 ± 3.5f
	B	4.1 ± 0.1cd	3.9 ± 0.1a	0.8 ± 0.1bc	0.7 ± 0.1a	30.4 ± 8.5cd	8.7 ± 1.2f *
MBA	MF	4.5 ± 0.3ab	4.0 ± 0.6a *	0.9 ± 0.0a	0.6 ± 0.1a *	43.1 ± 4.8a	28.6 ± 3.6ab *
	MC	4.5 ± 0.4a	3.8 ± 0.1a *	0.8 ± 0.1abc	0.7 ± 0.1a *	23.7 ± 7.4de	17.5 ± 4.2cde *
	MB	4.4 ± 0.1abc	3.9 ± 0.1a *	0.8 ± 0.1abc	0.7 ± 0.1a *	25.9 ± 5.5d	22.3 ± 2.2bc
	FC	4.2 ± 0.2a-d	3.8 ± 0.1a *	0.9 ± 0.1ab	0.7 ± 0.1a *	22.4 ± 3.4de	18.4 ± 4.9cde
	FB	4.2 ± 0.1bcd	3.8 ± 0.1a *	0.9 ± 0.1ab	0.7 ± 0.1a *	27.7 ± 1.3cd	18.9 ± 2.5cd *
	CB	4.2 ± 0.1cd	4.2 ± 0.1a	0.7 ± 0.1c	0.7 ± 0.1a	21.2 ± 4.7bc	12.2 ± 0.9def *
	MFCB	4.3 ± 0.1a-d	4.1 ± 0.1a	0.8 ± 0.1abc	0.7 ± 0.1a	20.2 ± 0.2e	30.8 ± 12.8a *
CK	CK	4.0 ± 0.1d	4.0 ± 0.2a	0.7 ± 0.01c	0.7 ± 0.0a	10.9 ± 1.4f	10.8 ± 0.1ef

Different lowercase letters denote significant differences among treatments at $p < 0.05$ on the same sampling date.

* means significant differences between 30d and 60d.

3.4. AcPase Activity and SAP Concentrations

Soil available phosphorus (SAP) concentrations and AcPase activity in the soil after a 60-day incubation are presented in Figure 2.3. The SAP levels of all treatments were very low, ranging from about 1 mg·kg^{-1} in CK to 5 mg·kg^{-1} in FB at 30 days (Figure 2.3a.). During the 60-day incubation, the SAP concentrations of all treatments increased at 30 days in contrast to CK, but significantly declined at 60 days (Table 2.4., $p < 0.01$). For example, the SAP in FB at 30 days was significantly higher than in other treatments, but then declined by about 63% at 60 days, which was in accord with the change in the corresponding AcPase activity (Figure 2.3b.).

Significant variations in AcPase activity in different treatments were detected ($p < 0.05$) and the impacts of incubation duration on AcPase activity in SBA were different from the effects in MBA (Table 2.4). For instance, AcPase activity in treatment M (belonging to SBA) showed no significant differences between 30 and 60 days, whereas AcPase activity in MFCB and FB (belonging to MBA) at 60 days showed lower values (Figure 2.4.). AcPase activity and SAPs in single applications of NFB (C, B, CB) were lower than in most of the other additions, although co-inoculation with both PSB and NFB (MC, FC, FB, MFCB) significantly increased the concentrations of SAP and AcPase activity at 30 days ($p < 0.05$, Figure 2.3.). However, this effect was minimal at 60 days. Compared to 30 days, the AcPase activity in FB at 60 days declined by 70%, which was accompanied by an obvious drop in the SAP concentration (Table 2.4, Figure 2.3; $p < 0.01$).

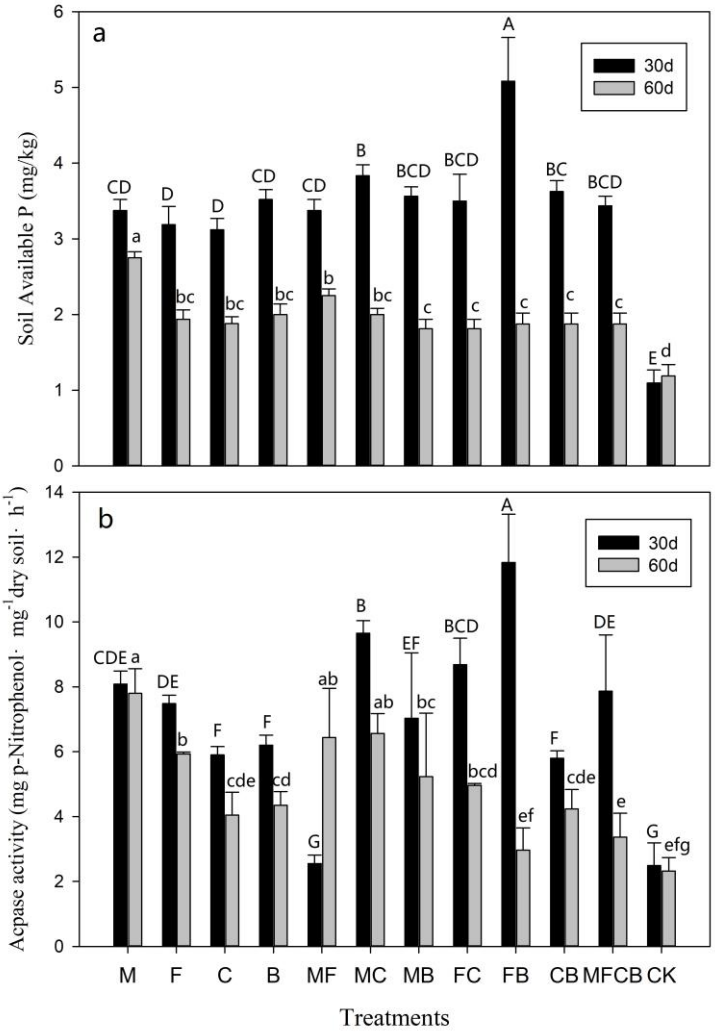


Figure 2.3. (a) Soil available phosphorus (SAP) contents and (**b**) AcPase activity of different treatments at the 30th (black bar) and the 60th day (gray bar). Marked capitals/lowercases above the standard line mean the significant difference among different treatments at the 30th day/the 60th day ($p < 0.05$).

3.5. Seedling Height and Biomass Accumulation

As shown in Figure 2.4a, the total biomass accumulation of *C. Paliurus* was significantly increased after bacterial additions. Plant biomass assessment was divided into four components, including stem, leaf, thick root and fine root. Compared with seedlings grown in native soil (CK), significant increments were detected in each component after bacteria addition. However, no positive effect of PSB application (treatment M and MF) on plant biomass was found during the investigation. On the contrary, the application of NFB (C and CB) significantly increased biomass accumulation of leaf and root. It is noteworthy that the biomass of above ground (stem, leaf) and thick root in co-inoculation with PSB and NFB (treatment MFCB) obtained about 47.8g and 20 g per plant respectively, which were significantly higher than when these microorganisms were used alone.

Compared with CK, the total increments of seedling height were improved after bacterial additions (Figure 2.4b). Specifically, dual inoculation with PSB (MF) and co-inoculation with PSB and NFB (treatment MFCB) resulted in greater influences on seedling height than other treatments, including treatment only retained with native microbes.

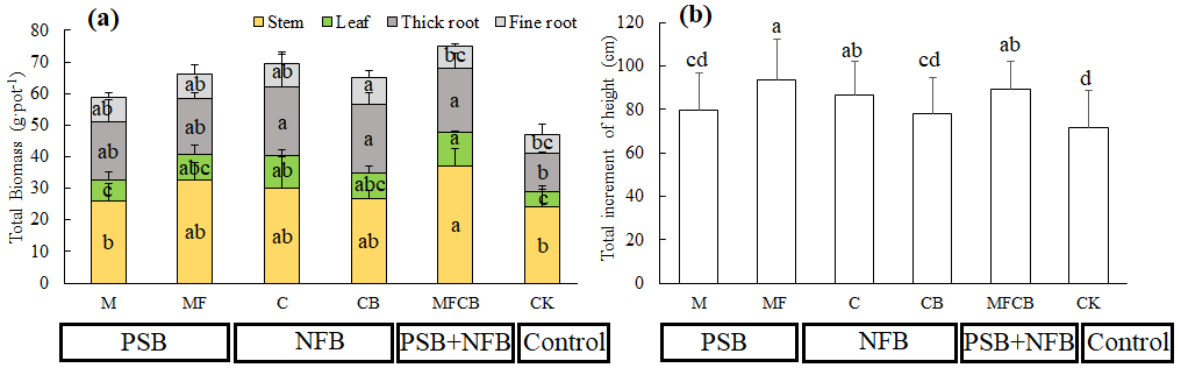


Figure 2.4. (a) Plant biomass (including stem, leaf, thick root, fine root) and (**b**) total increment of seedling height in different treatments with PSB (M and MF), NFB (C and CB) and PSB+NFB (MFCB).

4. Discussion

4.1. Changes of BQ Influenced by C Resources and Interactions of Bacteria

The available soil C, N, and P content are commonly in shortages in China's subtropical mountainous regions where the plant growth and production are limited. Bio-fertilization is a better choice for sustainably improving soil fertility compared to chemical fertilization (Igual et al. 2001, Gyaneshwar et al. 2002). Instead of making an artificial microcosm like previous studies (Debska et al. 2016, Yilmaz and Sonmez 2017), we incubated the inoculants in soil

that collected from natural fields with low-level C contents. Our results shows that soil biological properties, such as bacterial/fungal quantities and enzymes, are significantly correlated with soil C level (Tejada et al. 2008). As a result, BQ in most of the treatments performed similarly, which increases first then decreases during the 60-day incubation period (Figure 2.2). This indicates a coefficient restriction between limited C resources and the resilience of bacteria (Jing et al. 2017).

Here, three growth patterns of the inoculants were observed during the incubation, suggesting different responses of the BQ to single inoculant or in combination under C-deficient conditions (Figure 2.2). The BQ in some treatments increased again after their first peak, such as the co-inoculations (MF, FB, MFCB) in pattern 3 (Figure 2.2c). This was obviously different from previous publications in which only one peak was observed (Hameeda et al. 2008, Tahir et al. 2018, Valetti et al. 2018). We speculated that the occurrence of the second growth of bacteria was mainly stimulated by co-inoculation with PSB and NFB, where synergistic effects activated under the circumstances of limited available C and N resources in the microcosms (Wani et al. 2007, Yu et al. 2012). Similar studies reported that mixed microbial cultures allowed their components to interact with each other synergistically via physical or biochemical activities, thereby simultaneously improving viability (Vassilev et al. 2001, Shanmugam et al. 2014). In this experiment, synergistic mechanisms were found in the MF, FB, and MFCB, but BQ finally decreased under limited nutrients conditions. This result provides support when choosing the inoculant type (PSB+NFB) and frequency (30–45d) of fertilization when applying bio-fertilizer in such soils. Co-inoculation with PSB and NFB in soil results in more interactions of inoculants, such as the production of enzymes and organic acid, although more energy and inorganic nutrients would be consumed than when these organisms were used alone (Paerl and Pinckney 1996, Yu et al. 2012, Wei et al. 2018a). This was also supported by our study, where limited energy resources restrained the population growth for MBA at 30–40 days. Hence, the appropriate amount of C resource input during bio-fertilization is necessary when applying in such soil with low C level.

4.2. Additions of Bacteria Improved Soil Nutrients with Different Patterns

The responses of BQ to different inoculants under C-deficient conditions provided a better understanding of the relationship between the BQ, inoculant type, incubation duration, and available nutrients. Soil available nutrients, such as available N and P, are indispensable in regulating plant growth. However, soil available nutrients are often limited due to the changes in related enzyme and microorganism activities. During culture, the available nutrient contents in soil increased at an early stage (30 days) but declined at a later stage (60 days, Table 2.4; Table 2.5;

Figure 2.3). This pattern was consistent with the changing tendency of BQ (Table 2.2). Many studies have shown that the populations of beneficial microbes in soil provided the foundations that positively affected soil characteristics (Saxena et al. 2013, Rashid et al. 2016, Hu et al. 2017). Limited bacteria quantities in soil decreased available nutrients production, such as N and P, which may, in turn, restrict the population of microbes and affect the rates of the C decomposition process (Treseder 2008, Liu et al. 2012). Related reports have revealed that soil available C and N affect the pivotal process of microbial growth, and N-assimilation that driven by soil microorganisms mostly occurs in the NH_4^+–N of inorganic N and alanine of organic N (Hadas et al. 1992, Yang et al. 2016a, Wang et al. 2017). The microflora is positively correlated with soil C and available nutrients, and soil nutrients are conducive to increasing the abundance of soil microorganisms (Cai et al. 2015, Yang et al. 2016b). Thus, regular organic and bio-based fertilization of soils are favorable to the building of positive structures and functioning of the soil microbial community (Marschner et al. 2003, Frey et al. 2004, Tang et al. 2016).

Few effects of a single application of NFB on the availability of N were detected during culture (Table 2.5). However, co-inoculations with NFB significantly increased soil available P concentrations and the related enzyme activity (Figure 2.3a). Two assumptions to explain these synergistic effects are presented here: (1) co-inoculants with NFB could synergistically stimulate population growth of microbes based on the above discussion and (2) NFB could directly promote the activity of P-related enzymes (AcPase). Liu et al. stated that certain species of NFB could increase P uptake under N addition, which is related to soil P-related enzyme activity (Liu et al. 2013, Liu et al. 2017). AcPase activity is significantly affected by soil N, P conditions, and soil microorganism activities could result in an obvious change of AcPase activity. However, the AcPase activities of soil culture with NFB (C, B, CB) were obviously lower than the others (Figure 2.3b). This indicates the synergistic effect of specific NFB strains on SAP and related enzyme activity could be explained by stimulating growth and phosphate-solubilizing effects of PSB, rather than directly increasing the AcPase activity. This assumption could explain the result of the FB treatment, where the BQ decreased by about 72% at 60 days compared to 30 days, being accompanied by a drop in AcPase activity and SAP concentration.

The relationships between BQ and P-related indexes in SBA and MBA at 30d based on linear regression are shown in Figure 2.5. The P-related indexes (SAP and AcPase) significantly increased in both SBA and MBA with increases in the bacterial quantities ($p < 0.05$, Figure 2.5), while MBA resulted in higher value. And the SAP concentrations were correlated with the AcPase activity ($R^2 = 0.5423$, $p < 0.001$). This suggests that changes in the SAP concentrations mainly resulted from changes in the BQ and following altered P-related enzyme activities under

bio-fertilization.

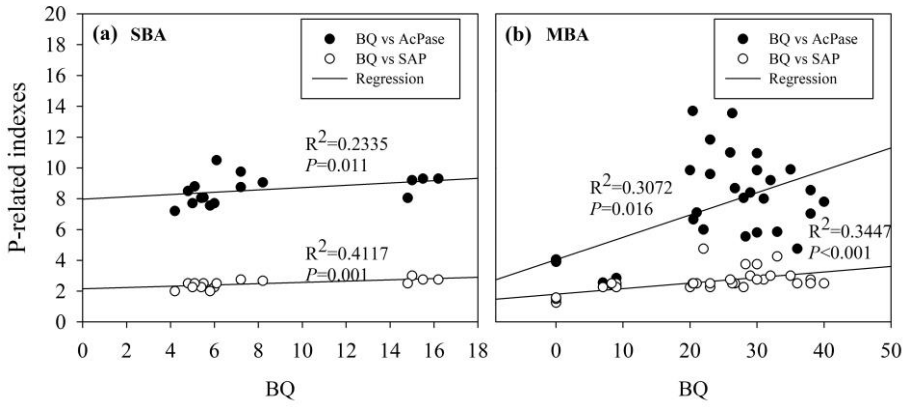


Figure 2.5. The relationships between (**a**) BQ vs P-related indexes in SBA at 30d, (**b**) BQ vs P-related indexes in MBA at 30 d on linear regression.

4.3. Co-inoculation with NFB and PSB resulted in higher plant biomass accumulation

The pot experiment was used for evaluating the pragmatic effects of these bacteria by compared to treatment with only native microbes (CK), while the lab experiment was conducted for verifying whether these bacteria could survive and benefit the soil nutrients. Similar researches were found in many published literature papers, in which sterilized or oven-dried soil was used for testing the effects of beneficial microorganisms without disturbance of other microbes under controlled conditions, and non-sterilized soil was used for investigating the pragmatic effects on plants under natural conditions (De Bolle et al. 2013, Park and Bolan 2013, Sanz-Saez et al. 2015, Porto et al. 2017). Plants accompanied by soil microorganisms in rhizosphere that could assist plants with nutrient acquisition (Hacquard et al. 2015). Therefore, additions of bio-fertilizer improve the available nutrients supply for plant growth. Under natural conditions, compared with treatment with only native microbes, soil nutrient contents, and plant N and P uptake were significantly improved after bacteria addition, especially for treatment MFCB (co-inoculation with PSB and NFB). More importantly, the relative abundances of these bacteria were increasing at the first 30 days, but decreased after that (data not shown). This suggests these bacteria could survive and enlarge population during the initial competition with native microbes, but continuous bio-fertilization is necessary to help these microorganisms get advantage. In the present study, bacteria addition increased seedling height and biomass accumulation under unsterilized soil condition. More importantly, they increased the biomass of the whole plant, especially the biomass of the leaf, which is the most valuable organ for medicinal use. Based on these results, applications of bio-fertilizer, such as MFCB, in leaf-use plantations of *C. paliurus* could be a potential sustainable

strategy for these plantations in the future.

To date, the interactive effects of co-inoculation with NFB and PSB on C-deficient soil conditions have been less studied. However, advanced mechanisms for interpreting the synergistic effects between NFB (*A. chroococcum*, *A. brasilence*) and PSB (*B. megaterium*, *P. fluorescens*) should be further investigated and evaluated to clarify the biochemical basis of these interactions. The survival and growth of strains vary with the chemical, physical, and biological differences between in vitro conditions and the field environment. A combination of NFB and PSB might cause competition for energy sources, such as root exudation and soil available nutrients. Hence, to obtain accurate conclusions about the effects of co-inoculation with NFB and PSB, further studies should be considered under different environmental media, and based on various research conditions (Yu et al. 2012, Debska et al. 2016).

5. Conclusions

Based on the results of the lab experiment and pot experiment, inoculation with beneficial bacteria had a positive effect on soil amendment and plant growth. Bacterial additions increased soil N and P availability, and co-inoculations with PSB and NFB enhanced the accumulation of the available P. However, decreases in soil nutrients were observed at 60 days compared to 30 days, which were induced by the decrease in bacterial quantities under C deficiency. These results highlight the interaction mechanism between strains and soil with the increase in the incubation duration under C-deficiency conditions. Besides, co-inoculations with PSB and NFB resulted in greater performance in plant growth promotion and nutrients uptake. In summary, aimed at amending the yellowish-brown clay soil with low levels of C, bacteria combinations (PSB+NFB) are recommended for practical application at intervals of 30–45 days. The lab experiment provided the basis for applying these microorganisms in natural environments, which helped us understand the possible interactions between PSB and NFB under C deficiency. The pot experiment results cross-validated that co-inoculation with PSB and NFB resulted in greater performance. This research gives the first interpretation of the mechanism of action and interaction between bacteria strains and soil under C deficiency, and contributes to the development of a biotechnological strategy, and sustainable agriculture, thereby minimizing the input of chemical fertilizers.

Chapter 3 Effects of phosphate-solubilizing bacteria and N_2-fixing bacteria on nutrient uptake, plant growth, and bioactive compound accumulation in *Cyclocarya paliurus* (Batal.) Iljinskaja

Adapted from: Wang Z, Chen Z, Xu Z, et al. Effects of phosphate-solubilizing bacteria and N_2-fixing bacteria on nutrient uptake, plant growth, and bioactive compound accumulation in *Cyclocarya paliurus* (Batal.) Iljinskaja[J]. Forests, 2019, 10(9): 772.

Abstract

Cyclocarya paliurus (Batal.) Iljinsk is a well-known medicinal plant as it accumulates bioactive compounds (BC), such as flavonoids, triterpenoids, and polysaccharides, in its leaves. However, the effects of plant growth-promoting rhizobacteria (PGPR) on the growth and BC yields in *C. paliurus* are not known. To fill this gap, the effects of different inoculants should be examined. A pot experiment was conducted and two-year-old *C. paliurus* seedlings were inoculated with three inoculant types: phosphate-solubilizing bacteria (PSB); N_2-fixing bacteria (NFB); and PSB+NFB. After four rounds of inoculation, the growth characteristics and concentrations of flavonoids, triterpenoids, and polysaccharides, as well as the nutrients in soil and leaves, were measured. *Results:* The inoculations resulted in the elevation of soil available nutrients, with improvements in plant growth, BC yield, and N and P uptake in leaves. Co-inoculation with PSB and NFB performed better in growth promotion and nutrient uptake than single bacterial inoculation. However, the changes in BC yields were mainly a result of elevated leaf biomass rather than BC concentrations, and leaf biomass was regulated by C:N:P stoichiometry. Co-inoculation with PSB and NFB was applicable for leaf production, while inocula related to NFB resulted in higher BC yields than PSB and control. *Conclusions:* Our results implied that bacterial inoculants improved plant growth and BC yield by altering the nutrients in soil and leaves, while three inoculant types showed a different pattern in which co-inoculation with four strains presented a greater performance than single bacterial addition.

1. Introduction

Cyclocarya paliurus (Batal.) Iljinsk, a deciduous tree, belongs to the family Juglandaceae and is mainly distributed across subtropical mountainous areas of China (Fang et al. 2011). Its leaves are often used in herbal tea (Kennelly et al. 1995) and as an essential ingredient of medicine to treat diabetes in China (Xie et al. 2015). A growing body of evidence indicates that diverse bio-activities (including antidiabetic, antioxidant, and antimicrobic activities) were found in the extracts of *C. paliurus* leaves (Zhang et al. 2010). These extracts are mainly comprised of flavonoids, triterpenoids, and polysaccharides, which contribute to protecting humans against chronic diseases (Xie et al. 2015, Wu et al. 2017). Based on these beneficial effects on human health, there is an increasing demand for the production of leaf and bioactive compounds (BC) in *C. paliurus* leaves for their medicinal applications.

As part of the Grain for Green Project (GTGP), the majority of *C. paliurus* plantations have to be assigned to poor sites in the mountainous areas in Southern China,. These regions are perceived to be infertile due to low levels of organic C and available nutrients (Fazhu et al. 2015, Huang et al. 2017), which are deemed to be the essential

nutrients for plant growth (Blaise et al. 2005). Chemical N and P fertilization are competent to promote plant growth and obtain optimal yield. Many types of studies have highlighted the positive effects of chemical fertilization on the yield and growth in medicinal plants. Deng et al. (Deng et al. 2012) reported that inorganic NPK fertilizer is conducive to optimizing the yields of targeted health-promoting substances in *C. paliurus*. Kumar et al. (Kumar et al. 2015) demonstrated that the highest seed yield and seed weight of fenugreek (*Trigonella foenumgraecum* L.) were found with chemical NPK fertilization at the rate of 50:50:25kg·ha^{-1}. However, after long-term chemical fertilization, soil degradation and pollution are getting worse. At the same time, limited nutrients in the soil are sustainably exploitable for plant uptake due to N-leaching, ammonia volatilization, and P-immobilization (Gyaneshwar et al. 2002, Kaur and Reddy 2015). Recently, owing to advances in the understanding of microorganism–plant interactions, researchers' attention has been attracted by increasing applications of biological and natural fertilizers, because of their outstanding performance in crop growth and smaller ecological footprint compared with chemical fertilizers.

Of the recommended strategies, the utilization of bio-fertilizer based on plant growth-promoting rhizobacteria (PGPR) has proven to be an efficient and eco-friendly management practice (Vessey 2003). These bio-fertilizers contain living beneficial microorganisms that can colonize the rhizosphere and stimulate crop growth by increasing the supply of available nutrients to the host plant when applied to the soil (Vessey 2003). PGPR, such as N_2-fixing bacteria (NFB) and phosphate-solubilizing bacteria (PSB), have already been sufficiently studied. For instance, *Azotobacter chroococcum* and *Azospirillum brasilence,* two free-living aerobic NFB can be found in most soil and have the ability to convert inert N_2 into available forms for plants (Kizilkaya 2008). *Bacillus megaterium* and *Pseudomonas fluorescens* (PSB) are notable for the ability to solubilize unavailable phosphates in soil, as well as produce a wide variety of metabolites like auxin (Kang et al. 2014, Dadrasan et al. 2015). The application of PGPR as a bio-fertilizer on medicinal seedlings has resulted in a higher yield of BC and plant growth in different crops, such as *Glycyrrhiza uralensis* Fisch (Xie et al. 2018b), *Juglans regia* L. (Yu et al. 2012), and *T. foenumgraecum* L. (Dadrasan et al. 2015). Some researchers have proven that mixed inoculation of PSB and NFB was an alternative bio-fertilizer for supplying N and P to walnut plants (Yu et al. 2012). However, there is no information about the effects of bio-fertilizer, especially for co-inoculation with PSB and NFB, on plant growth and BC of *C. paliurus.*

The BC in this study included total flavonoid, total triterpenoid, and water-soluble polysaccharide in *C. paliurus* leaves. Among flavonoids, seven flavonoid monomers were identified in the previous study (Cao et al. 2017) and presented important values for medicinal use (Spencer et al. 2004, Rajendran et al. 2014), thus were chosen in this

study. The aim of this study was to investigate the effects of PSB (*B. megaterium* and *P. fluorescens*), NFB (*A. chroococcum* and *A. brasilence*), and co-inoculation with PSB and NFB accompanied with organic fertilizer, on the growth characteristics, nutrients in soil and leaves, and the yield and concentration of BC in *C. paliurus* leaves. We hypothesized that, (1) PGPR inoculated in the rhizosphere can facilitate plant growth and BC yield of *C. paliurus,* (2) such a promotion may directly or indirectly derive from altered internal C: N: P stoichiometry in leaves, (3) co-inoculation with PSB and NFB will result in greater performance than when these strains were used alone. Our findings build the connection between PGPR and plant secondary metabolites and offer opportunities to choose a sustainable way to reform the soil and establish *C. paliurus* plantation for pharmaceutical supply.

2. Materials and Methods

2.1. Seedlings, Growth Media, and Microorganism's Preparation

On November 1, 2017, two-year-old *C. Paliurus* seedlings were chosen from Muchuan, Sichuan, China (28°96′ N, 103°98′ E), based on the previous research (Liu et al. 2018c). The initial heights of the seedlings ranged from 32–38.5 cm and the ground caliper ranged from 5.02–6.1 mm.

The medium for plant growth in pot-experiment was a mixture of soil, sand, organic fertilizer and coconut residuum (7:2:0.8:0.2, v/v). The soil was collected from the plow layer of soil (0–20 cm) at *C. Paliurus* plantation in Nanjing, China (31°35′ N, 119°10′ E), more information was presented in our previous study (Wang et al. 2019b). The organic fertilizer added to the medium was used to improve the survival and multiplication of bacteria. One seedling was planted in each pot (top diameter: 25 cm, bottom diameter: 20 cm, height: 30 cm) containing 5 kg of growth medium. The basic physicochemical properties of medium were as follows: pH 5.98, total C of 18.9 $g \cdot kg^{-1}$, total N of 0.79 $g \cdot kg^{-1}$, total P of 0.30 $g \cdot kg^{-1}$, total of K 0.10 $g \cdot kg^{-1}$, available N of 12.68 $mg \cdot kg^{-1}$, and available P of 5.56 $mg \cdot kg^{-1}$.

The bacterial strains used in this study were *Bacillus megaterium* W17 (Yu et al. 2012), *Pseudomonas fluorescens* W12 (Yu et al. 2011), and *Azotobacter chroococcum* HKN-5 (Wu et al. 2005) and *Azospirillum brasilence* CW903 (Kim et al. 2005). These bacteria have been documented with the ability of improving soil nutrients, such as N and P, and none of these bacterial strains showed any antagonistic effects against one another (Wang et al. 2019b). Prior to use, bacteria strains were incubated in lysogeny-broth medium (LB, pH 7.0, comprised of 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) to the mid-exponential growth phase. At the same time, the bacterial

population was examined in a lab using the plate count serial dilution method (Sanders 2012) while experimenting on building a standard curve between optical density and bacterial quantities. After that, the inoculants were diluted by sterile LB medium to a final concentration of 1×10^8 colony forming units (CFU)·mL^{-1} according to the standard curve.

2.2. Site Description and Experimental Design

The seedling nursery was located in Lishui, Nanjing, China (31°35′ N, 119°10′ E), where the *C. paliurus* plantation was established. This area is a typical transition zone from the north subtropics to the subtropics, where the climate is mild and humid, with abundant rainfall (1037 mm/year) and sunshine (2146 h/year), the annual average temperature is about 15.4 °C.

The experiment was laid out in a three-block pattern based on randomized complete block design. Seven treatments included three inoculant types (PSB, NFB, PSB+NFB), and two control (without bacteria but LB medium and water), each treatment contained 60 seedlings that were equally divided into three blocks. Details are shown in Table 3.1. After seedlings were well established, bio-fertilization with seven treatments were conducted four times with the interval of about 45 days (April 4, May 19, July 6, and August 19, 2018, respectively). Specifically, 50 mL (1×10^8 CFU·mL^{-1}) inoculations in total were circularly injected into rhizosphere in each pot according to bio-fertilization regimes in Table 3.1.

2.3. Measurement of Soil Available Nitrogen and Phosphorus

For the measure of soil available N (SAN) and soil available P (SAP) in the rhizosphere, five soil samples (5–10 cm) were collected randomly for each treatment on September 8, 2018, and kept at 4 °C prior to analysis. SAN (NH_4^++NO_3^-) was determined by extraction with 2M KCl in 1:5 (w/v) soil-to-solution ratio, shaking for 1 h at 200 rpm, and followed by quantification using a continuous flow analyzer (Bran + Luebbe AA3, Germany). SAP was extracted by ammonium fluoride and hydrochloric acid in 1:10 (w/v) and determined using the molybdenum-blue method (Olsen 1954).

Table 3.1. Fertilizing doses of seven bio-fertilization regimes (mL·pot^{-1}).

Inoculant type	Treatment	***M:*** *Bacillus megaterium*	***F:*** *Pseudomonas fluorescens*	***C:*** *Azotobacter chroococcum*	***B:*** *Azospirillum brasilence*	LB	water
PSB[1]	M	50	0	0	0	0	0
	MF	25	25	0	0	0	0
NFB[2]	C	0	0	50	0	0	0
	CB	0	0	25	25	0	0
PSB+NFB[3]	MFCB	12.5	12.5	12.5	12.5	0	0
Control[4]	LB	0	0	0	0	50	0
	CK	0	0	0	0	0	50

[1] Phosphate-solubilizing bacteria (PSB): inoculated with strain *B. megaterium* (M), *B. megaterium*, and *P. fluorescens* (MF). [2] N_2-fixing bacteria (NFB): inoculated with strain *A. chroococcum* (C), *A. chroococcum*, and *A. brasilence* (CB). [3] PSB+NFB: co-inoculation with four strains. [4] Control: inoculated with LB medium and water

According to a previous study (Wang et al. 2019b), the bacterial population hit a peak at days 30-45 of incubation. Available soil N and P contents and related enzyme activity were significantly increased in co-inoculations with PSB and NFB. Hence, the bio-fertilization frequency (every 45 days) and inoculants types (Table 3.1) were determined in this pot-experiment based on previous results.

2.4. Plant Growth and Leaf Harvest

Seedling height and caliper were measured for all healthy seedlings (about 27 seedlings for each treatment) at every fertilization time, and the total increment of growth was calculated by the difference of initial and final height/caliper. For biomass measurement, three seedlings of each treatment were excavated entirely on September 6, 2018, washed and separated into four components (leaf, stem, thick root, and fine root). Afterward, all components were dried at 60 °C and weighed, respectively. The total dry mass of each seedling was calculated as the sum of leaf, stem, and root dry weight. The ratio of underground biomass to above-ground biomass (root/shoot ratio) was calculated.

After biomass assessment, all the leaves of *C. Paliurus* (three samples of each treatment) were ground and stored at room temperature for the following measurement of nutrients and bioactive compounds in leaves.

2.5. Measurement of Total Carbon, Nitrogen, and Phosphorus in Leaves

For the measurement of total carbon (C) and nitrogen (N) contents, each sample (50.0 mg) of leaves was wrapped up with a tin can, and total C and N were determined by the elemental analyzer (vario MAX CN, Elementar, Hanau, Germany). For the measurement of total phosphorus (P) contents, each sample (1 g) was digested by HNO_3 and $HClO_4$ (5:1 in volume), and total P was determined by the molybdenum-blue method.

2.6. Extraction and Determination of Bioactive Compounds

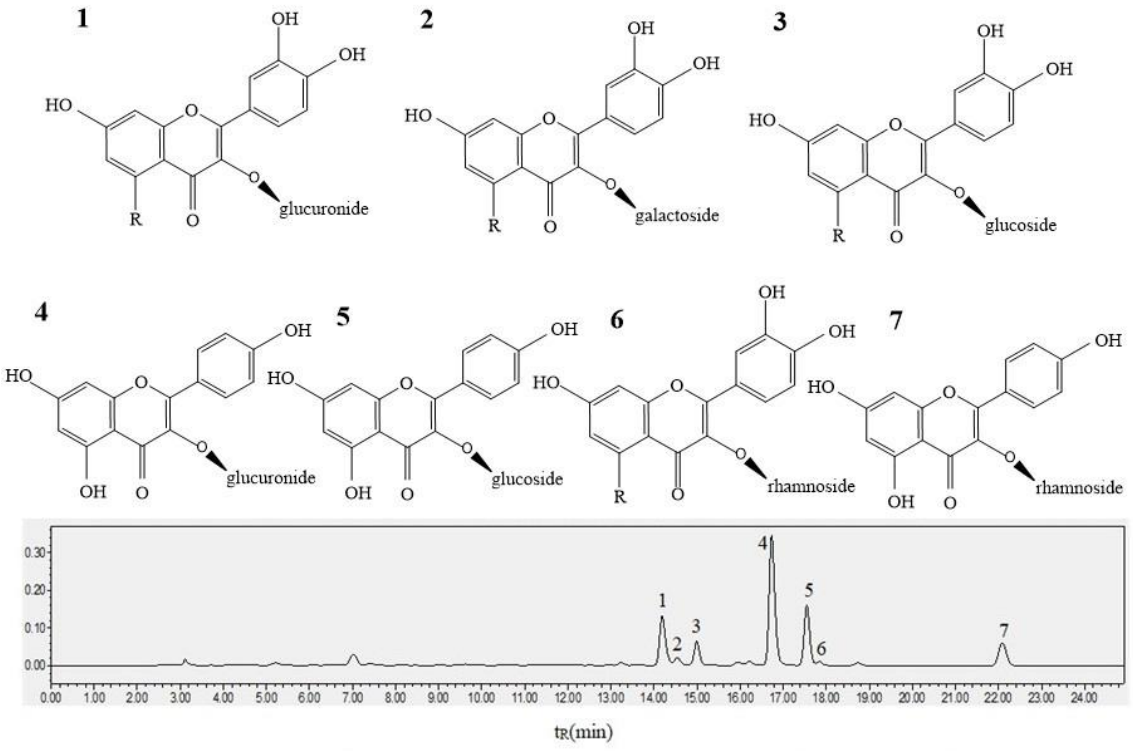
Flavonoids were extracted from *C. paliurus* leaves using an ultrasonic-assisted method with 75% ethanol after removing fat-soluble impurities with petroleum ether. The total flavonoid concentration was determined using a colorimetric method with detection at 415 nm (Bao et al. 2005) and was calculated using the standard Rutin curve and expressed as a milligrams Rutin equivalent per gram of dry mass (mg/g). Seven flavonoid monomers (Figure 3.1), including quercetin (quercetin-3-O-glucuronide; quercetin-3-O-galactoside; quercetin-3-O-rhamnoside), kaempferol (kaempferol-3-O-glucuronide; kaempferol-3-O-glucoside; kaempferol-3-O-rhamnoside), and isoquercitrin, were determined and identified by high-performance liquid chromatography system (HPLC, Waters, Milford, MA, USA) coupled with quadrupole time-of-flight mass spectrometry (HPLC-Q-TOF-MS) (Cao et al. 2017). The extraction of water-soluble polysaccharide in *C. paliurus* leaves was carried out as described previously by Fu et al. (Fu et al. 2015) and the polysaccharide concentration was determined by the phenol–sulfuric acid method. For triterpenoid extraction, 2.0 g of leaves were extracted using an ultrasonic-assisted method. Briefly, 50 mL of 75% ethanol was added to each sample, and the extraction was conducted for 45 min at 65 °C and repeated twice. The total triterpenoid concentration was determined according to a previously described laboratory procedure using a colorimetric method with slight modifications (Fan and He 2006).

The yields of these bioactive components in leaves were calculated as the concentration multiplied by the biomass of leaves.

2.7. Statistical Analysis

The Shapiro–Wilk test and Levene's test were used to test the normal distribution of data and homogeneity of variances, respectively. When there were significant effects ($p < 0.05$), Duncan's multiple range test was applied to determine the differences among individual treatment means. Tamhane's T_2 was used to test for differences

among treatments when variances of tested data were not equal. All statistical analyses were considered significant at $p < 0.05$. The pairwise correlations of plant growth characteristics, nutrient uptake, concentrations, and yields of bioactive components were elucidated using Spearman's correlation analysis. All statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA).



Peak No.	t_R(min)	[M-H]-	MS/MS Fragment Ion (m/z)	Formula	Identification
1	14.4	477.0673	301.0351;178.9979;151.003	$C_{21}H_{18}O_{13}$	quercetin-3-O-glucuronide
2	14.7	463.0888	301.0343;271.0244;178.9979;151.0029	$C_{21}H_{20}O_{12}$	quercetin-3-O-galactoside
3	15.2	463.0888	301.0338;271.0239;178.9981;151.0029	$C_{21}H_{20}O_{12}$	isoquercitrin
4	16.9	461.9727	286.0431;285.0401;113.0235;85.0296	$C_{21}H_{18}O_{12}$	kaempferol-3-O-glucuronide
5	17.7	447.0935	285.0388;284.0319;255.0295	$C_{21}H_{20}O_{11}$	kaempferol-3-O-glucoside
6	18.0	447.0973	301.0337;285.0388;255.0294;217.0133	$C_{21}H_{20}O_{11}$	quercetin-3-O-rhamnoside
7	22.3	431.0981	285.0394;255.0295;227.0342	$C_{21}H_{20}O_{10}$	kaempferol-3-O-rhamnoside

Figure 3.1. High-performance liquid chromatography (HPLC) chromatogram of treatment MFCB and identified the chemical structure of the seven flavonoids monomers.

3. Results

3.1. Soil Available N and P

The contents of soil available N (SAN) and soil available P (SAP) in the rhizosphere are presented in Figure 3.2. After four rounds of bio-fertilization, SAN and SAP were significantly increased compared to the control; however,

different patterns were noted. Dual inoculation with two NFB (treatment CB) resulted in the highest contents of SAN and showed an obvious advantage over other inoculants. On the other hand, the highest content of SAP was observed in co-inoculation with PSB and NFB (treatment MFCB), while single inoculation (treatment M and C) caused lower effects.

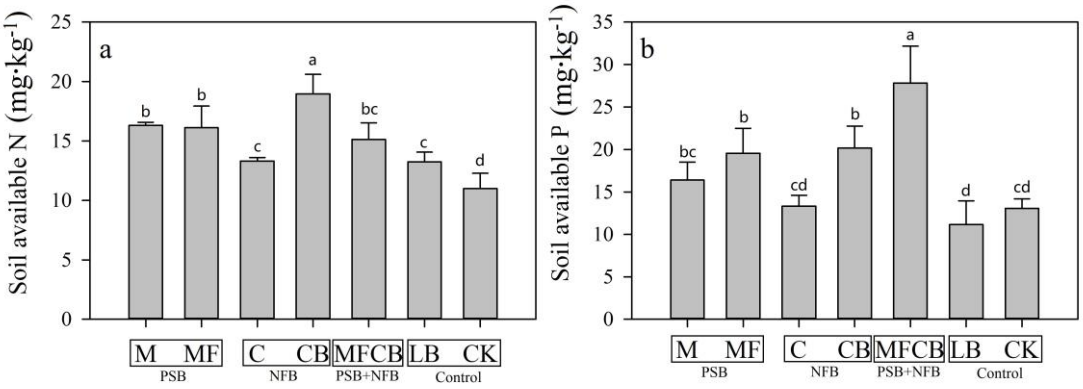


Figure 3.2. Contents of (**a**) soil available N, and (**b**) soil available P in the rhizosphere as affected by different inoculant types (PSB, NFB, PSB+NFB). Different lowercase letters denote significances of soil available N and soil available P among treatments at $p<0.05$ level.

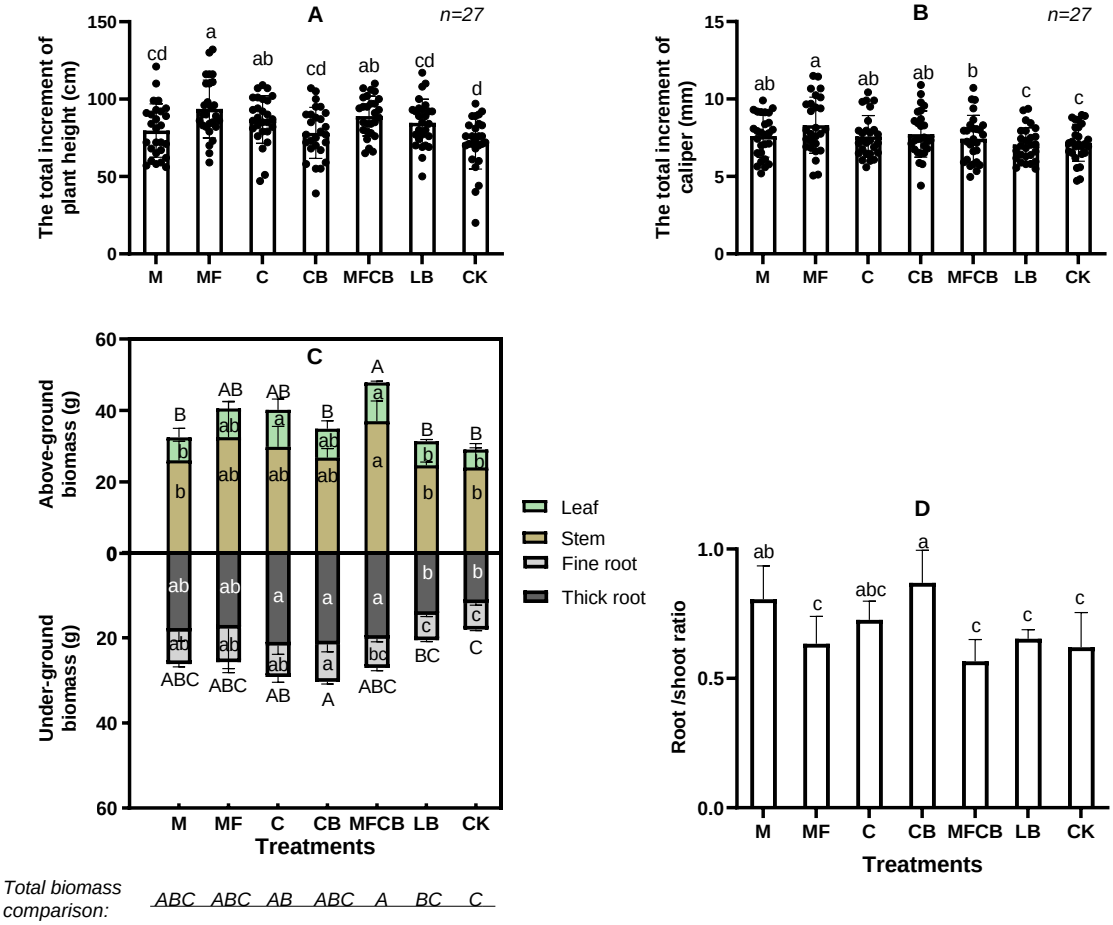


Figure 3.3. Plant growth, biomass and partitioning of *C. paliurus* as influenced by inoculants. (**A**): The total increment of plant height. (**B**): The total increment of the caliper. (**C**): Comparison of different biomass components (leaf, stem, fine root, and thick root) and above/under/total biomass among all treatments; different lowercase letters inside the bar denote significant variances of biomass component among treatments at $p<0.05$; different capital letters above/under the bar denote significant differences of above/under-ground biomass between treatments at $p<0.05$; the comparison of total biomass presented at the bottom. (**D**) Root/shoot ratio was calculated as the ratio of underground biomass to ground biomass. Different lowercase letters above the bar in **A**, **B**, and **D** denote significant differences among treatments at $p<0.05$.

3.2. Plant Growth and Biomass

The bio-fertilization had a significant effect on plant growth (Figure 3.3A and B) and biomass production (Figure 3.3C). The total increment of plant height ranged from 71.81 cm in CK to 93.67 cm in MF, and the level of treatment MF, C, and MFCB were significantly higher than the control (LB and CK) ($p < 0.05$). Significant increments of seedling caliper were noted for all bio-fertilizer treatments, ranged from 7.05 mm in CK to 8.31 mm in MF. For both height and caliper, the highest increments all occurred in MF, reaching 93.67 cm and 8.31 mm, respectively.

By compared with CK and LB, co-inoculation with PSB and NFB (treatment MFCB) resulted in the highest total biomass accumulation (74.9 g per plant) and higher ground biomass distribution (47.83 g), while the application of NFB (C and CB) significantly increased the underground biomass. However, no positive effect of PSB application (M and MF) on plant biomass was found during the investigation (Figure 3.3C). Consequently, the root/shoot ratio was significantly increased in CB and decreased in MFCB, respectively (Figure 3.3D). Noteworthily, the MFCB treatment significantly increased the leaf biomass accumulation, which is the target production for food and medicinal use.

3.3. C: N: P Stoichiometry in C. paliurus Leaves

The contents of C, N, and P in *C. paliurus* leaves for each treatment were measured, and the C/N, C/P, and N/P ratios were calculated (Table 3.2). According to the results, the N and P contents in leaves were increased in three inoculant types (PSB, NFB, PSB+NFB) compared to the control ($p<0.05$), ranging from 21.00–27.81 $g \cdot kg^{-1}$ (N) and from 1.57–1.95 $g \cdot kg^{-1}$ (P), respectively. Co-inoculation of PSB and NFB resulted in higher N and P contents in leaves than single bacterial addition (M and C, $p<0.05$). However, the dual inoculation of two PSB (MF) or two NFB (CB)

possessed no significant advantage over single bacteria.

On the other hand, the applications of three inoculant types caused a slight but nonsignificant increment of C contents in leaves (ranging from 455.9–465.8 g·kg^{-1}). As a result, the C/N ratios and C/P ratios of controls (LB and CK) were significantly higher than all treatments with inoculations, while N/P ratios indicated a contrary pattern.

Table 3.2. Contents of total carbon, nitrogen, phosphorus, and their ratios in *C. paliurus* leaves.

Inoculant type	Treatment	Carbon (g·kg^{-1})	Nitrogen (g·kg^{-1})	Phosphorus (g·kg^{-1})	C/N	C/P	N/P
PSB	M	464.28a[1]	23.04d	1.86b	20.18b	250.14cd	12.41d
	MF	455.90a	23.82cd	1.76c	19.16bc	258.50bc	13.5ab
NFB	C	464.19a	25.83b	1.91ab	17.97c	243.42de	13.54ab
	CB	459.50a	25.08bc	1.73c	18.33c	265.69b	14.50a
PSB+NFB	MFCB	464.23a	27.81a	1.95a	16.70d	237.63e	14.23ab
Control	LB	465.78a	21.44e	1.62d	21.73a	287.04a	13.23cd
	CK	462.08a	21.00e	1.57d	22.00a	295.26a	13.42cd

[1] Different lowercase letters in the same column denote significant differences among treatments at $p<0.05$ level

3.4. Flavonoids

Concentrations and yields of seven flavonoid monomers and total flavonoid in *C. paliurus* leaves are presented in Figure 3.4. Total flavonoid concentrations were slightly elevated ($p>0.05$) after bio-fertilization and ranged from 19.0 mg·g^{-1} in M to 23.23 mg·g^{-1} in CB, while a significant increment of total flavonoid yield was observed in all treatments except M (Figure 3.4F). Furthermore, inocula related to NFB (C, CB, MFCB) resulted in higher yields than PSB and the control.

In terms of the seven flavonoid monomers, significant variances of concentrations and yields were detected among all treatments (Figure 3.4a–g). However, the accumulation of flavonoid monomers showed different variation patterns between PSB, NFB, and PSB+NFB. The co-inoculation of PSB and NFB (MFCB) improved the accumulation of monomers in both concentration and yield, while PSB had negative effects. On the other hand, inoculation with NFB possessed a significant advantage over inoculation with PSB. The highest concentration and yield of all flavonoid monomers were observed in kaempferol-3-O-glucuronide in MFCB, which obtained 2.0 mg·g^{-1} and 21.6

mg·plant^{-1}, respectively (Figure 3.4d).

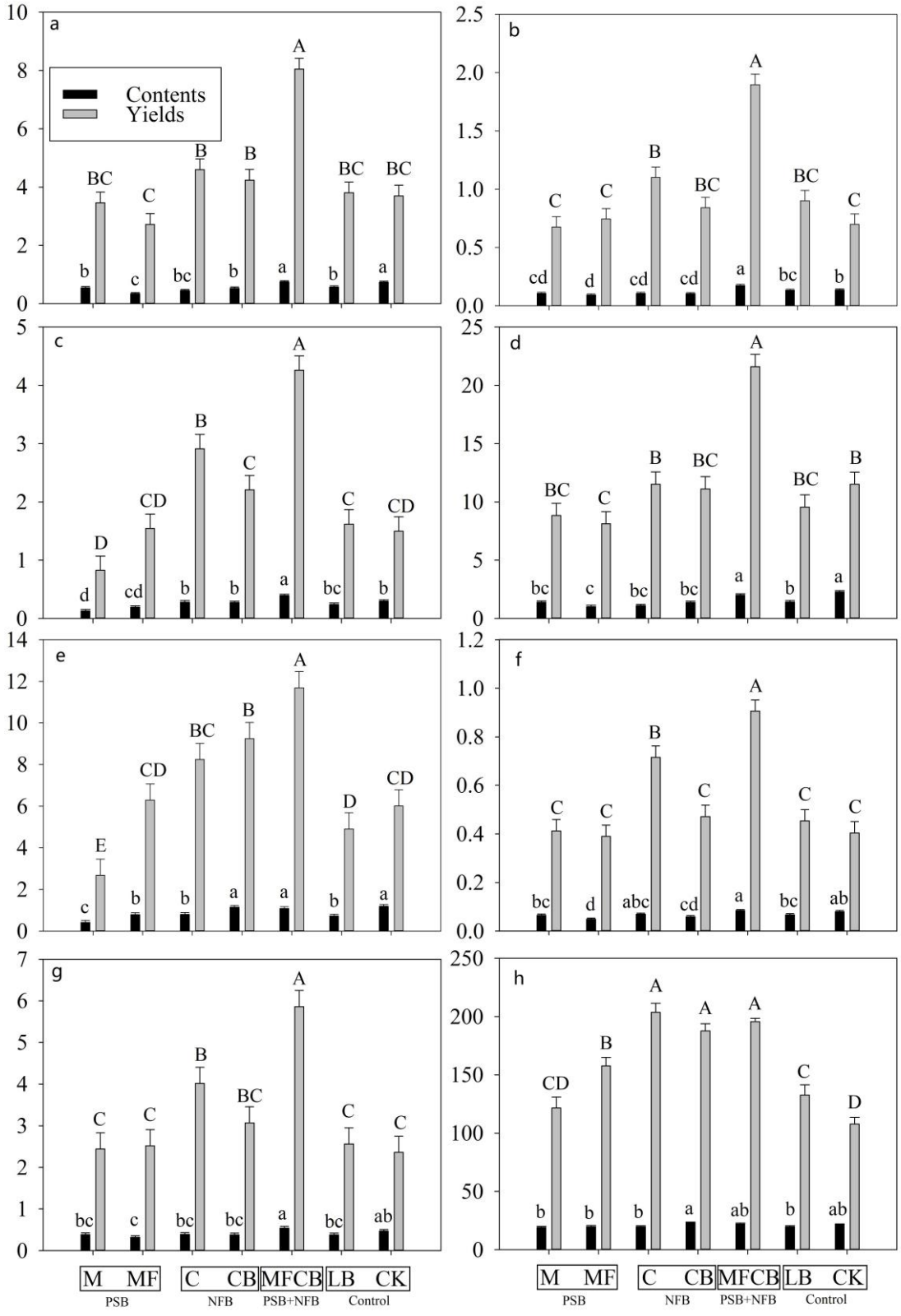


Figure 3.4. Concentrations (mg·g^{-1}) and yields (mg·plant^{-1}) of seven flavonoid monomers and total flavonoid. (**a**):

quercetin-3-O-glucuronide; (**b**): quercetin-3-O-galactoside; (**c**): isoquercitrin; (**d**): kaempferol-3-O-glucuronide; (**e**): kaempferol-3-O-glucoside; (**f**): quercetin-3-O-rhamnoside; (**g**): kaempferol-3-O-rhamnoside; (**F**): total flavonoid. Same as follows. Different lowercase/capital letters denote significant differences in concentration/yield among treatments at $p<0.05$.

3.5. Water-soluble Polysaccharide and Triterpenoid

The effects of bio-fertilization on water-soluble polysaccharide and triterpenoid concentrations in *C. paliurus* leaves were not significant ($p > 0.05$). However, inocula related to NFB resulted in higher yields of polysaccharide and triterpenoid than PSB and the control. The highest yield of total triterpenoid and polysaccharide in *C. paliurus* leaves were achieved in treatment with C, followed by MFCB, whereas the lowest yield was noted in CK. Compared with CK, total triterpenoid yields in treatment C and MFCB increased by 81.6% and 63.6%, while the polysaccharide yields increased by 103.9% and 84.7% respectively.

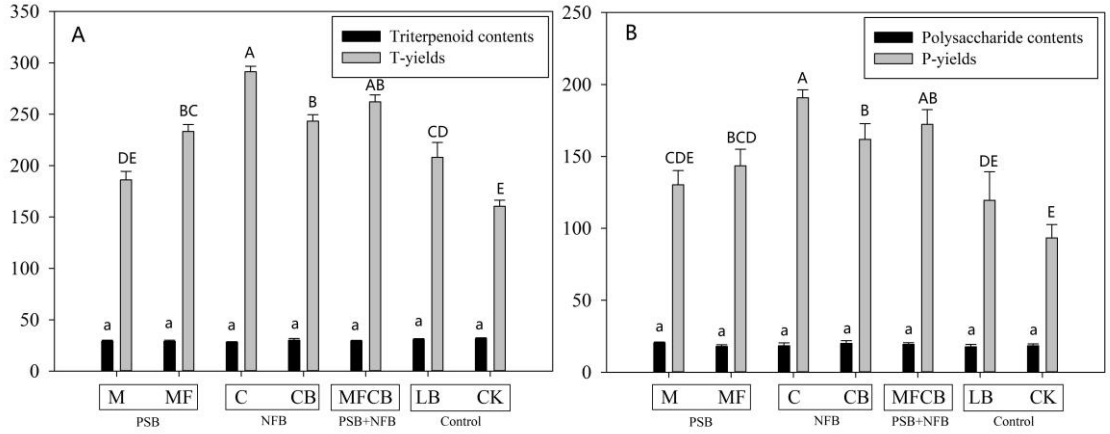


Figure 3.5. Concentrations ($mg \cdot g^{-1}$) and yields ($mg \cdot plant^{-1}$) of total triterpenoid (**A**) and water-soluble polysaccharide (**B**). Different lowercase/capital letters denote significant differences of concentrations/yields among treatments at $p<0.05$.

4. Discussion

To increase the medicinal values of *C. paliurus*, optimizing production of the plantation is a research focal point, especially in cultivation management strategies, such as chemical fertilization, light quality, and artificial shade (Deng et al. 2012, Liu et al. 2018b, Deng et al. 2019a). As a sustainable method for amending the soil, PGPR were applied extensively in crop growing and have been proven to have positive effects on crop output as well as soil

properties (Kaur and Reddy 2015, Xie et al. 2018b). In this study, we focused on not only biomass improvement (leaf) but also the accumulation of BC in *C. paliurus* leaves by the addition of PGPR to the growing medium.

4.1. PGPR symbiosis increased nutrients in soil which improved plant growth

As a multifunctional medicinal plant, *C. paliurus* leaf is the principal organ for accumulating bioactive compounds (BC) (Deng et al. 2019b). Moreover, as the organ of photosynthesis, nutrients in leaves directly or indirectly affect C assimilation, phytochemical accumulation, and plant growth. Of all nutrients, N and P are indispensable in regulating plant growth and ecosystem productivity (Elser et al. 2007, Vitousek et al. 2010). However, available nutrients are often limited under poor soil conditions, which could be amended by bio-fertilizers.

Usually, the promotions in growth and biomass are supposed to derive from improved soil available nutrients after bacterial inoculation, so as to promote N and P uptake in the plant (Megali et al. 2014). This is supported by our results that SAN, SAP, and N and P contents in leaves were increased under three inoculant types (Figure 3.2; Table 3.2). According to the correlation analysis, N and P contents in leaves were significantly correlated with SAN, SAP, and growth characteristics (Table S2, $p < 0.05$). Therefore, the improvement of plant growth mainly resulted from elevated N and P uptake, which were induced by increased nutrients in the soil. Similar results were reported in different plants, whose growth characteristics and biomass correlated with the internal nutrients uptake altered by PGPR (Singh and Kapoor 1998, Gull et al. 2004).

For the response of improvement in growth to PSB and NFB inoculation, the widely accepted speculation is that plant growth and biomass accumulation would be affected by C: N: P stoichiometry, induced by fertilization, photosynthesis, and microorganisms (Lillo et al. 2008, Elser et al. 2010). As shown in Table 3.2, Table S2, and Figure S1-c, d, the leaf biomass was positively correlated with both N and P contents in leaves ($p < 0.05$), and negatively correlated with C/N and C/P ratio ($p < 0.05$), while no significant correlations were observed between leaf biomass and N/P ratio. Clearly, PGPR are responsible for facilitating N and P availability, trigging N and P uptake by the host plant, then regulating plant growth and biomass accumulation by altering the internal nutrient balance (Peng et al. 2019).

4.2 PGPR additions improved the BC output mainly by increasing the leaf biomass rather than concentrations

Main BC in *C. paliurus* leaves, such as flavonoids, triterpenoids, and polysaccharides are responsible for numerous medicinal effects. Much literature has concluded that the accumulation of these BC in *C. paliurus* leaves was

influenced by genetic, cultivation practices and climatic factors (Deng et al. 2012, Fu et al. 2015, Liu et al. 2018b, Liu et al. 2018c). Among these, fertilizations play vital roles in the oriented cultivation of *C. paliurus* plantation for medicinal use.

It is known that plant secondary metabolites could be induced by adverse environmental conditions and regulated by internal nutrients balance (Lillo et al. 2008). Previous studies indicated that C, N, S, and P contents in plants were related to both primary growth and secondary metabolites (Gigolashvili and Kopriva 2014, Canovas et al. 2018). Xie et al. reported that the improved root P status to arbuscular mycorrhizal fungi could affect plant C balance and induce more C partitioning to secondary metabolism (Xie et al. 2018a). Plants accompanied by soil microorganisms could be assisted with nutrient acquisition, while N and P uptake could affect the allocation of C resources and cause changes in C: N: P stoichiometry (Zhao et al. 2015). These changes were considered as the nutritional benefits of PSB and NFB symbiosis to host plants, and affected primary growth as well as secondary growth (Singh and Kapoor 1998, Vessey 2003).

As presented in this study, the yield of total flavonoid, polysaccharide, and triterpenoid was significantly elevated under inocula related to NFB (C, CB, MFCB), while there was little influence on their concentrations (Figure 3.4F). This is in accord with the results of regression analysis, in which N and P contents in *C. Paliurus* leaves were positively correlated with leaf biomass and yields of BC, but there were no significant correlations with concentrations (Figure S1). Bio-fertilization is in favor of plant primary growth but not the accumulation of BC. Thereby, the increments of the yield of BC mainly resulted from the promotion of leaf biomass rather than their concentrations.

In contrast, for the seven flavonoid monomers, significant variances in both concentrations and yields were detected among all treatments (Figure 3.4a-g). However, we found only the concentration of isoquercetin was significantly correlated with N uptake, while other monomers indicated no significance (Table S1). Hence, different PGPR, such as PSB and NFB, may indirectly influence the biosynthesis of flavonoids through manipulating other factors, such as gene expression (Lillo et al. 2008), enzyme activity (Dastmalchi et al. 2017, Deng et al. 2019b), or phytohormone (Salla et al. 2014). For all flavonoid monomers, they possess the common biosynthetic pathway with little difference. Flavonoids are usually conjugated with glucose and biosynthesized from phenylalanine and malonyl-CoA produced by the shikimate pathway in plant (Iwashina and Kitajima 2000). Increased nutrients uptake in plants could contribute to the production of the precursor, such as phenylalanine, which is the common precursor of primary metabolism and secondary metabolism (Schmidt et al. 2010).

Plant growth and biomass accumulation mainly depend on primary metabolism, while plant defense and adaptation rely on secondary metabolism (Tavarini et al. 2018). Many theories have been proposed to explain potential trade-offs between plant primary growth and secondary metabolite synthesis (Cai et al. 2009). It worth noting that economic returns may not increase with a higher concentration of secondary metabolites in plants, as a higher concentration is often offset by lower biomass under stress conditions (Afshar et al. 2015). Thus, to achieve a high yield of objective ingredients, cultivation practices in soil/media is required, but the relationship between leaf production and phytochemical concentration in leaves should be balanced when the plantation is used for medicinal production.

4.3 Selections of PGPR could be considered for multiple purposes of *C. paliurus* plantation

The effects of bio-fertilization depend on plants, soil types, and harvest targets (Bhardwaj et al. 2014). As a multi-functional woody plant, *C. paliurus* could be utilized for timber, tea, as well as medicine (Fu et al. 2015). Although inoculations resulted in increments of plant growth, the effects of PSB and NFB differed on growth regulations and accumulations of BC. For timber use, a fertilization strategy in favor of vegetative growth, reflected in tree height, diameter, and volume of timber, should be considered as a priority. As shown in our work, MFCB and MF treatments improved growth and above-ground biomass accumulation of *C. paliurus* under yellowish-brown clay soil mixed with organic fertilizer (Figure 3.3). Hence, treatment MF and MFCB are alternatives in plantation for timber use.

Different from plant growth in most crops, more attention should be paid to harvesting a high yield of BC for medicinal plants, such as *C. paliurus*. However, fewer effects of fertilization on concentrations of medicinal components were reported (Dadrasan et al. 2015, Deng et al. 2019b), as revealed in our study. Similarly, biomass improvement of the main organ for the collection of medicinal components by fertilizers could achieve a high yield of target components. Moreover, our results (Figure 3.3) and predictions (Table S3) proved the feasibility of fertilizers.

In addition, the selection of PGPR should be considered according to the soil conditions and harvest targets. As found in *C. paliurus*, inocula related to NFB (C, CB, MFCB) resulted in higher BC yields than PSB and the control, while the highest production of leaves was in MFCB, twice as much as the control (Table S3).

No matter what *C. paliurus* plantation is focused on, soil conditions should be taken into account. The present study found that co-inoculation with PSB and NFB resulted in higher SAP than the others, while treatment CB

achieved the highest value of SAN. Based on our previous study, synergistic effects between PSB and NFB may contribute to higher availability of soil nutrients and stimulate plant growth (Wang et al. 2019b). Several studies reported that inoculating plants with both PSB and NFB could result in higher available N and P contents in soil and nutrient uptake in plants (Yu et al. 2012, Kumar et al. 2017). This is because mixed microbial cultures allowed their components to interact with each other synergistically via physical or biochemical activities, thereby simultaneously improving viability in soil (Shanmugam et al. 2014).

5. Conclusions

In this study, PGPR inoculations resulted in a significant increment of soil nutrients, with an improvement in plant growth, biomass, and N and P uptake in *C. paliurus* leaves. Co-inoculation with PSB and NFB presented better performances than single-bacterial addition. Significant influences of PGPR on the concentrations of flavonoid monomers were noted, while no effects were found in the concentrations of bioactive compounds. The changes in bioactive compound yields were mainly a result of leaf biomass promotion rather than their concentrations, and leaf biomass was regulated by C:N:P stoichiometry in leaves. Co-inoculation with PSB and NFB was more appropriate for leaf production, while inocula related to NFB resulted in higher bioactive compound yields than PSB and the control. This study firstly interpreted nutritional mechanisms involved in growth regulation and phytochemical accumulation of *C. paliurus* under bio-fertilization and provided selections of PGPR for multiple purposes of *C. paliurus* plantation. Future research should focus on non-nutritional mechanisms involved in PGPR symbiosis affecting secondary metabolite accumulation.

Supplementary material

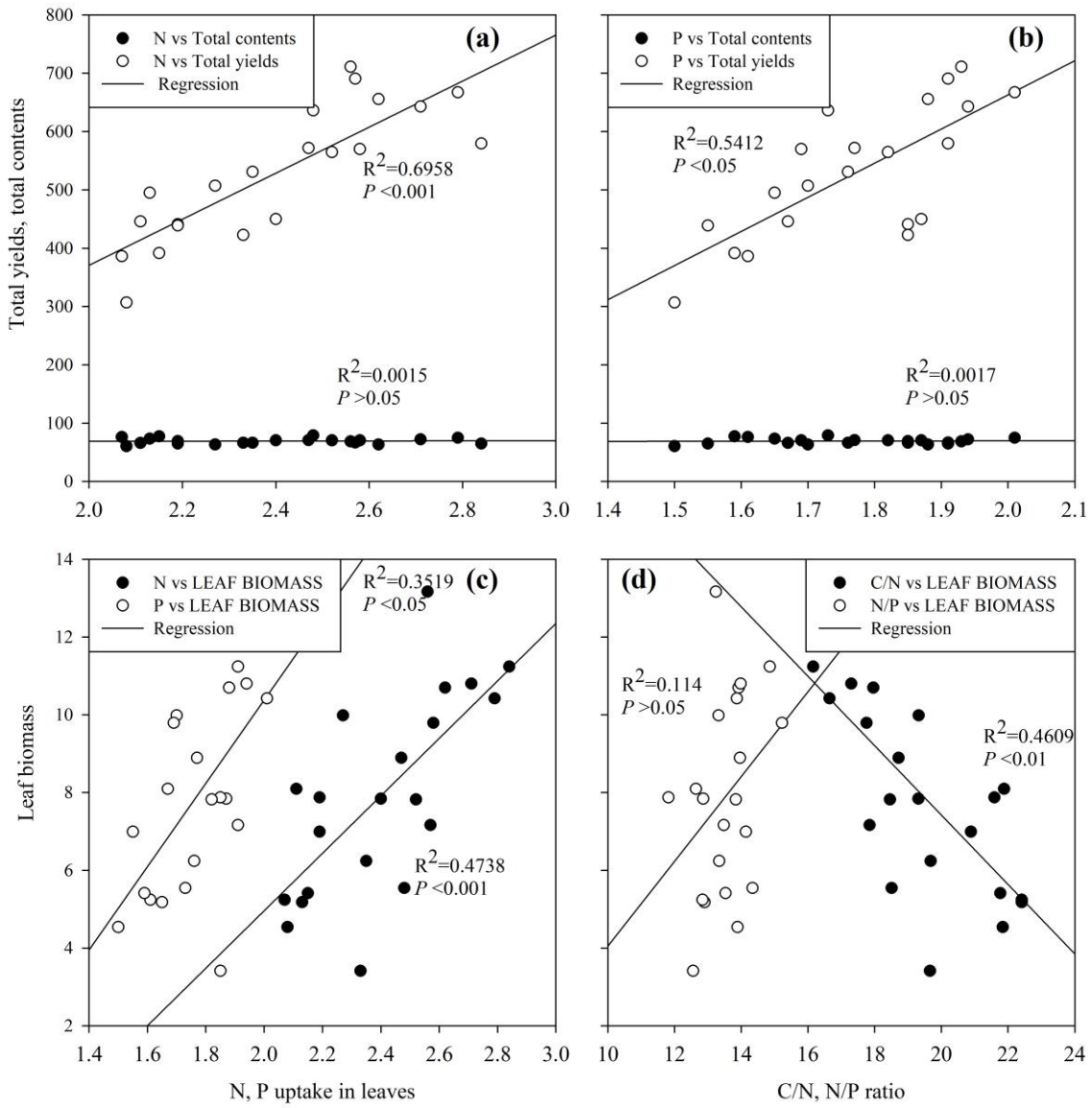


Figure S1. The relationships between N, P contents in leaves vs total yields/contents of bioactive compounds, and C: N: P vs leaf biomass. (**a**) N contents in leaves vs total yields/contents of bioactive compounds, (**b**) P contents in leaves vs total yields/contents of bioactive compounds, (**c**) leaf biomass vs N/P contents in leaves (**d**) leaf biomass vs C/N and N/P ratio in leaves on linear regression (n=21). Total contents/yields are calculated as the sum of total flavonoid, triterpenoid and polysaccharide contents/yields.

Table S1. Spearman's correlations (r value) between C, N, P uptake and contents/yields of bioactive compounds.

Variables	C	N	P	C/N	C/P	N/P
a	-0.02	-0.14	-0.13	0.1	0.16	0.15
Ya	-0.09	0.66**	0.50*	-0.67**	-0.44*	0.58**
b	0.01	0.02	-0.03	-0.05	0.04	0.2
Yb	-0.06	0.68**	0.49*	-0.68**	-0.44*	0.53*
c	-0.04	0.44*	0.19	-0.47*	-0.12	0.62**
Yc	-0.02	0.72**	0.50*	-0.71**	-0.42	0.61**
d	-0.09	-0.21	-0.25	0.16	0.25	0.13
Yd	-0.14	0.48*	0.33	-0.53*	-0.29	0.53*
e	-0.08	0.23	-0.09	-0.27	0.16	0.64**
Ye	-0.12	0.71**	0.41	-0.73**	-0.34	0.70**
f	0.05	0.02	0.1	-0.05	-0.08	0.05
Yf	-0.03	0.66**	0.53*	-0.65**	-0.46*	0.47*
g	-0.07	0.19	0.17	-0.24	-0.16	0.21
Yg	-0.04	0.83**	0.68**	-0.83**	-0.61**	0.54*
F	-0.06	0.31	0.12	-0.36	-0.11	0.43*
YF	-0.06	0.82**	0.65**	-0.82**	-0.62**	0.49*
T	0.33	-0.08	-0.13	0.11	0.19	0.08
YT	0.15	0.83**	0.71**	-0.79**	-0.65**	0.45*
P	0.62**	0.04	0.24	0.01	-0.17	-0.25
YP	0.21	0.81**	0.74**	-0.77**	-0.67**	0.32

a: quercetin-3-O-glucuronide; b: quercetin-3-O-galactoside; c: isoquercitrin; d: kaempferol-3-O-glucuronide; e: kaempferol-3-O-glucoside; f: quercetin-3-O-rhamnoside; g: kaempferol-3-O-rhamnoside. Ya-Yg means yields of seven flavonoid monomers; YF, YT, and YP mean yields of total flavonoids, triterpenoid and polysaccharide * means correlation is significant at $p<0.05$; ** means correlation is significant at $p<0.01$, n=21.

Table S2. Spearman's correlations (r value) between C, N, P uptake, soil available nutrients, and plant growth characteristics.

Variables	Soil available N	Soil available P	Biomass				Height	Caliper
			Stem	Leaf	Thick root	Fine root		
C	-0.12	0.24	-0.19	0.08	-0.23	-0.48*	0.09	-0.2
N	0.53*	0.81**	0.35	0.71**	0.43	0.17	0.52*	0.35
P	0.50*	0.76**	0.38	0.66**	0.43*	0.06	0.55**	0.28
CN	-0.52*	-0.80**	-0.37	-0.71**	-0.46*	-0.27	-0.48*	-0.36
CP	-0.48*	-0.69**	-0.37	-0.63**	-0.46*	-0.1	-0.55**	-0.31
NP	0.20	0.41	0.19	0.32	0.18	0.27	0.05	0.07

Table S3. Predication of bioactive compounds yield and leaf production of 2-year-old *C. paliurus* under same bio-fertilizer treatments[1].

Inoculant type	Treatment	flavonoids (g·10000 seedlings^{-1})[2]	triterpenoid (g·10000 seedlings^{-1}) [2]	polysaccharide (g·10000 seedlings^{-1}) [2]	leaf production (kg·10000 seedlings^{-1}) [2]
PSB	M	1215.8cd	1861.6de	1302.0cde	63.7c
	MF	1575.5b	2332.1bc	1434.3bcd	80.1bc
NFB	C	2035.4a	2914.0a	1908.2a	103.4ab
	CB	1875.5a	2432.9b	1619.0b	80.7bc
PSB+NFB	MFCB	1955.5a	2620.6ab	1722.8ab	108.2a
Control	LB	1325.6c	2079.6cd	1194.2de	67.5c
	CK	1077.6d	1604.6e	933.2e	50.6c

Note: [1] The same treatments include seedlings age and provenance, bio-fertilization rate and amount, and growth media. [2] Bioactive compounds yield and leaf production of *C. paliurus* are calculated based on this pot experiment result.

Chapter 4 Succession of the resident soil microbial community and plant growth dynamics in response to periodic inoculations

Wang Z, Chen Z, Kowalchuk G A, et al. Succession of the resident soil microbial community in response to periodic inoculations. Applied and Environmental Microbiology, 2021, 87:e00046-21.

Abstract

To maintain the beneficial effects of microbial inoculants on plant and soil, repeated inoculation represents a promising option. Until now, the impacts of one-off inoculation on the native microbiome have been explored, but it remains unclear how long and to what extent the periodic inoculations would affect the succession of the resident microbiome in bulk soil. Here we examined the dynamic responses of plant growth, soil functions and resident bacterial community in the bulk soil to periodic inoculations of phosphate-solubilizing and N_2-fixing bacteria alone or in combination. Compared to single-strain inoculation, co-inoculation better stimulated plant growth and soil nutrients. However, the benefits from inoculants did not increase with repeated inoculations and were not maintained after transplanting to a different site. In response to microbial inoculants, three patterns of shifts in bacterial composition were observed – fold increased, fold decreased, and resilience. The periodic inoculations impacted the succession course of resident bacterial communities in bulk soil, mainly driven by changes in soil pH and nitrate, resulting in the development of three main cluster types throughout the investigation. The single and mixed inoculants transiently modulated the variation in the resident community in association with soil pH and C/N, but finally the community established and showed resilience to subsequent inoculations. Consequently, the necessity of repeated inoculations should be reconsidered, and while the different microbial inoculants showed distinct impacts on resident microbiome succession, communities ultimately exhibited resilience.

1. Introduction

Soil microorganisms are the main drivers of soil ecosystem functioning, including the mineralization of organic matter, nutrient cycling and resistance to soil-borne diseases (Mendes et al. 2011, Thiele-Bruhn et al. 2012, Zhong et al. 2020). However, the native soil microbial community is sensitive to exogenous disturbances due to anthropogenic activities (fertilization, pesticide application, irrigation) and natural climate change (temperature, rainfall) (Hartmann et al. 2015, Suleiman et al. 2016). The impacts of abiotic disturbances, such as chemical fertilization and water stress, on soil microorganisms have been widely reported (Evans and Wallenstein 2014, Zhou et al. 2015). In addition, soil resident microbial communities are frequently subjected to biotic disturbances such as application of biocontrol or beneficial microbial inoculants, and naturally occurring microbial disturbance such as soil-borne pathogens (van Elsas et al. 2012, Mallon et al. 2015). These invading microbes, whether beneficial microbial inoculants for promoting plant productivity or harmful pathogens affecting plant health, can

alter microbial community succession, composition and diversity (Xiong et al. 2017, Lourenco et al. 2018).

The host plants can assemble beneficial microorganisms in the rhizosphere via signals such as root exudates in response to attack by soil-borne pathogens (Berendsen et al. 2012). As a manual and sustainable soil management strategy, microbial inoculant is efficient and eco-friendly for improving crop productivity and soil properties, with living beneficial microorganisms colonizing the rhizosphere and increasing nutrients availability to the host plant (Vessey 2003, Pagnani et al. 2020). Several studies have explored the influence of one-off microbial inoculation on soil nutrients, plant growth and defense to pathogens (Megali et al. 2014, Yilmaz and Sonmez 2017, Berg et al. 2020). However, these beneficial effects are frequently restricted due to many factors, e.g., soil nutrient (Treseder 2008) and organic matter content (Tejada et al. 2008), seasonal variation (Banik et al. 2019), and competition with resident microbiota (Cipriano et al. 2016). To achieve sustained benefits on soil properties and plant growth, periodic applications of microbial inoculants might be helpful. However, not all invasive microbes can successfully join the resident community, soil resources and the composition of native community determine resilience and resistance to intruders (Saison et al. 2006).

Disturbances are often classified as pulse (short-term) or press (continuous or long-term) depending on their duration and influence on the soil properties (Bender et al. 1984). Although beneficial microbial inoculants can be effective remediation agents in soil, successive inoculation may act as a press disturbance that directly or indirectly disrupts the native soil microbial habitat (Li et al. 2017a, Lourenco et al. 2018). Press disturbances of soil microbial communities due to long-term inorganic or organic fertilization have been reported for a wide range of locations and crop types (Calleja-Cervantes et al. 2015, Mbuthia et al. 2015, van der Bom et al. 2018), but little information is available on the response of the soil resident microbial community to repeated inoculant input. Previous studies (van Elsas et al. 2012, Mallon et al. 2018) suggested that a single microbial invasion may alter the resident community composition, functioning, as well as nutrient niche breadth, and that microbial diversity determines the outcome of biotic invasions, but the extent and persistence of the influence of periodic microbial inoculations on shifts in native communities remain unclear. Mawarda et al. (2020) also indicated that deliberate release of microbial inoculants may cause resource competition, synergism, and antagonism effects on resident microbiome. Given the growing use of such practices, it is important to understand the underlying mechanisms of the responses of the microbial community under different inoculant additions in order to evaluate soil quality and resilience (Qiao et al. 2019).

The influences of different microbial inoculants on soil properties under controlled conditions and the practical

effects on plant nutrient uptake under natural conditions have been thoroughly evaluated (Wang et al. 2019b, Wang et al. 2019c, Wang et al. 2021b). In this study, we sought to investigate the dynamics of soil nutrients, plant growth and the soil resident bacterial community in response to successive microbial inoculations over the course of a growing season. We hypothesized that inoculations would increase soil nutrient availability as well as plant growth, and that these beneficial effects would increase along with repeated applications. We hypothesized that repeated inoculations would act as press disturbances and affect the stability of soil resident microbes and modulate the composition of the soil resident microbiome. These disturbances would lead to different patterns of bacterial community shifts. Moreover, we hypothesized different inoculants could be associated with disparate impacts on the resident microbiome, host plant growth and soil function.

The present experiment was conducted from November 2017 to October 2018 in pots planted with the native medicinal plant *Cyclocarya paliurus* (Batal.) Iljinsk (Fang et al. 2011). Four plant-beneficial strains were applied alone or in combination for four times with an interval of 45 days. An afforestation experiment was subsequently established in 2019 using the same inoculated seedlings, to evaluate the following effects of past microbial inoculations on plant growth at a different site. The plants and bulk soils were dynamically sampled throughout the study period to (i) investigate the soil functioning and dynamic growth of plant under different inoculant types and different time points, (ii) evaluate the shifts in the native microbial community in response to periodic inoculations, (iii) identify the changing patterns of microbial taxa and the differences between different inoculation types, and (iv) analyze the underlying biotic and abiotic factors in shaping the soil microbial community.

2. Materials and methods

Site description and material preparation

The seedling nursery site was a semi-automatic plant growth unit located in Baima, Nanjing, China (31°35′ N, 119°10′ E), while the afforestation site was located at the Jiangsu Traditional Chinese Medicine (TCM) Science and Technology Park, Taizhou, China (32°37′ N, 119°98′ E). These sites (115 km distance apart) are in the typical transition zone from the north subtropics to the subtropics and have the same soil type (clay loam soil), abundant rainfall (1037 mm/year) and sunshine (2146 h/year), and an annual average temperature of approximately 15.4 °C. The soil properties of Baima are pH 5.98, total C of 18.9 $g \cdot kg^{-1}$, total N of 1.61 $g \cdot kg^{-1}$, total P of 0.42 $g \cdot kg^{-1}$, available N of 12.68 $mg \cdot kg^{-1}$, and available P of 5.56 $mg \cdot kg^{-1}$, whereas in Taizhou, the soil properties are pH 7.31, total C of

12.72 g·kg^{-1}, total N of 0.88 g·kg^{-1}, total P of 0.45 g·kg^{-1}, available N of 88.35 mg·kg^{-1}, and available P of 32.22 mg·kg^{-1}.

Four beneficial strains, including *Bacillus megaterium* W17, *Pseudomonas fluorescens* W12, *Azotobacter chroococcum* HKN-5, and *Azospirillum brasilense* CW903, were used alone or in combination in this study. Our previous study monitored the effects of single and mixed inoculants on soil properties and their survival dynamics in the soil (Wang et al. 2019b), thus provided a reference for selecting appropriate microbial inoculants and inoculation period for this study. According to their survival abilities and effects on soil, we selected single inoculants (M: inoculated with *B. megaterium*; C: inoculated with *A. chroococcum*) and mixed inoculants (MF: inoculated with both *B. megaterium* and *P. fluorescens*; CB: inoculated with both *A. chroococcum* and *A. brasilense*; MFCB: co-inoculation with all four strains). These bacteria have been documented to improve soil nutrients status and do not have antagonistic effects on one another (Wang et al. 2019b). Each strain was grown in lysogeny broth medium at 28 °C, shaking at 180 rpm for 24–26 h until an optical density (OD) of 0.9 at 600 nm, which corresponded to the log phase. The bacterial population was examined in a lab using the plate count serial dilution method while experimenting on building a standard curve between optical density and bacterial quantities. The suspensions were adjusted to a final concentration of 1×10^{8} colony forming units (CFU)·mL^{-1} for each strain based on OD_{600nm}.

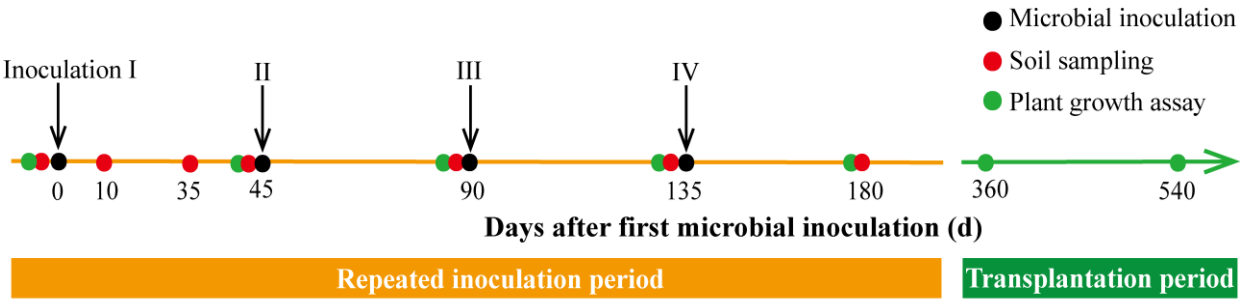


Figure 4.1 Timeline for microbial inoculation, soil sampling, and plant growth measurement. The major part of the experiment was conducted in 2018 in Nanjing (inoculation period). After that, seedlings were transplanted to Taizhou in 2019 (transplantation period).

Experimental design and soil sampling

The pot experiment was laid out in a three-block pattern based on a randomized complete block design with five inoculant types (M, MF, C, CB, MFCB). The non-inoculated samples served as control in this study because our previous study has indicated that the single addition of growth medium did not significantly impact plant growth, biomass, and nutrient acquisition compared to non-inoculated samples (Wang et al. 2019c). Each treatment

consisted of 60 *C. paliurus* seedlings that were equally divided into three blocks. The container seedlings were transplanted to the pots at November 1, 2017, and each seedling pot (top diameter: 25 cm, bottom diameter: 20 cm, height: 30 cm) contained 5-kilogram soil as growth media. Inoculations were conducted four times with an interval of approximately 45 days (April 4, May 19, July 6, and August 19, 2018), with the same dose each time (5×10^9 cell per plant) (Figure 4.1). Briefly, we dug a 5-cm-depth circle around the pot (near the edge of plant root) for all seedlings (including CK) to access the lateral root. Then, 50 mL of inoculum was injected into each circle which was subsequently covered by soil. After that, all inoculated seedlings including pot soils were transplanted to a different site (Taizhou) in March, 2019 with the same experimental design, to evaluate the legacy effects of past inoculations on plant growth.

For each treatment in each block, five bulk soil samples (0-10 cm) were randomly collected and equally mixed into one sample, resulting in a total of three samples for each treatment in three blocks. The sampling method was as described previously (Wang et al. 2019b). Briefly, five to eight random vertical holes (diameter: 8 mm; depth: 60 mm) were implemented by sampling tube for each pot to lessen the disturbance of sampling on microbes, this provided about 50-gram soil for each duplicate of each treatment. The sampling times were the day before the first inoculation (0d), 10 days after the first inoculation (I-10), 30 days after the first inoculation (I-30), 45 days after the first inoculation (I-45), 45 days after the second inoculation (II-45), 45 days after the third inoculation (III-45), and 45 days after the fourth inoculation (IV-45) (Figure 4.1). The bulk soil samples were split into two parts, one was stored at 4 °C prior to the analysis of biochemical properties, and the other was stored at -20 °C prior to DNA extraction.

Plant growth measurements

Plant growth was evaluated as seedling height and ground diameter, which were measured for all healthy seedlings before the first inoculation and 45, 90, 135, 180, 360, and 540 days after the first inoculation (Figure 4.1). The mean relative growth rate in height (RGRh) and ground diameter (RGRd) were also calculated as described by Mazarura et al. (Mazarura et al. 2013). The equations are shown below, where h_i or d_i is the initial growth in height (cm) or ground diameter (mm) and h_f or d_f is the final height (cm) or ground diameter (mm), $t_2 - t_1$ represents the time difference (d) between initial and final sampling date.

(1) $\mathrm{RGRh} = \frac{\log_e h_f - \log_e h_i}{t_2 - t_1}$

(2) $\mathrm{RGRd} = \frac{\log_e d_f - \log_e d_i}{t_2 - t_1}$

Soil biochemical properties

Soil biochemical properties included soil pH, C/N ratio, contents of soil alkali-hydrolyzable nitrogen (SAN), inorganic nitrogen (SIN), and available phosphorus (SAP), and the activity of phosphatases and nitrogenase. Soil pH was determined by a pH electrode (IQ 160 pH Meter, Spectrum Technologies, Inc., America) with a soil-to-water ratio of 1:2.5. The total C and N contents were determined using an elemental analyzer (Vario MAX CN, Elementar, Hanau, Germany). SAN content was quantified by the method of Roberts et al. (Roberts et al. 2011). SIN content (KCl extractable NH_4^+ and NO_3^-) was analyzed by extraction with 2M KCl in a soil-to-solution ratio of 1:5 (w/v) with shaking for 1 h at 200 rpm, followed by quantification using a continuous flow analyzer (Bran + Luebbe AA3, Germany). SAP was extracted by 1:10 (w/v) ammonium fluoride and hydrochloric acid and determined using the molybdenum-blue method (Olsen 1954). Acid phosphatase activity was assessed using the method described by Tabatabai and Bremner (Tabatabai and Bremner 1969). Soil nitrogenase activity was measured by the acetylene reduction method (David et al. 1980).

DNA extraction and Illumina MiSeq sequencing

Soil total DNA (0.5 g soil) was extracted using the NucleoSpin® Soil DNA Kit (Macherey-Nagel GmbH & Co.KG, Düren, Germany), according to the manufacturer's protocols. The final DNA concentration and purity were determined by a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with primers 515F (5'-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT -3') in a thermocycler PCR system (GeneAmp 9700, ABI, USA). PCR was carried out under the following conditions: initial denaturation at 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s, and a final extension at 72 °C for 7 min. PCR amplifications were performed in triplicate in a 20-μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The PCR amplicons were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), triplicate PCR amplifications for each sample were conducted and pooled as a PCR product, and then sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, California, USA), according to the

standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) (Liu et al. 2020c).

To minimize the effects of random sequencing errors, raw fastq files were quality-filtered by Trimmomatic (Bolger et al. 2014) and merged by FLASH (Magoč and Salzberg 2011) with the following criteria: (i) reads were truncated at any site with an average quality score <20 over a 50-bp sliding window; (ii) sequences whose overlap was longer than 10 bp were merged according to their overlap with no more than 2 bp mismatch; (iii) sequences of each sample were separated according to barcodes (exact match) and primers (allowing 2 nucleotide mismatch), low-quality and ambiguous reads (sequence shorter than 150 bp) containing ambiguous bases were removed. Chimeras were identified and removed with the UCHIME algorithm (Edgar et al. 2011). Operational taxonomic units (OTUs) were clustered at 97% similarity using UPARSE (v.7.1) and were declared invalid if fewer than four sequences were detected in one sample. The sampling effort was estimated by Good's coverage (Table S1). The Silva database (132/16S bacteria) was used with a minimum percent identity threshold of 70% for taxonomic assignment. Singletons were removed prior to further analysis. Mothur (v.1.30.2) was used to calculate bacterial α-diversity indices (Shannon, Simpson, Chao, and ACE) to estimate bacterial diversity and richness.

Bioinformatics and statistical analyses

Statistical analyses including multiple comparisons for plant growth and soil nutrient variables were performed using the SPSS software (v.20.0, SPSS Inc., USA). Two-way analysis of variance (ANOVA) was applied to analyze the effects of different inoculants and different sampling time on the plant height and ground diameter in Baima. One-way ANOVA was applied to evaluate the effects of different treatments on the plant height and ground diameter in Taizhou. Student's T-test was used to compare the difference of the same treatment between Baima and Taizhou. For sequence data, each sample was rarefied to 36,281 sequences before the alpha diversity analyses (Table S1), which included Good's coverage, observed OUT numbers, the ACE and Chao1 richness indexes, and the Shannon and Simpson diversity indexes. Analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were performed to evaluate significant differences in microbial community composition among the six inoculation treatments. Microbial community type analysis was conducted to evaluate the dynamic shifts in microbial community structure during the 180-day investigation (Wu et al. 2011). Briefly, according to the relative abundance of bacteria at the phylum level, the Jensen-Shannon Distance (JSD) was calculated and clustered by the partitioning around medoids (PAM), the optimal clustering K value was calculated by the Calinski-Harabasz (CH) index, PCoA (principal coordinate analysis) was performed based on Bray–Curtis distances, and the coordinates

were used to visualize differences in microbial community structure. The Pearson correlation coefficient was calculated by a cor() function using the microbiome data from each time point and visualized by using the corrplot package (Zhang et al. 2018), the significance level was tested by cor.mtest() function.

Heatmaps were generated based on the 50 most abundant taxa at the family level, to output the dynamic shifts of soil resident community composition under different inoculants. The taxa clusters were conducted based on abundance similarities between each group in vegan package. To explore the biological factors involved in the differences between the clusters derived from microbial community type analysis, we used Linear discriminant analysis effect size (LEfSe) analyze to identify taxonomic markers at phylum level for three main clusters in inoculated samples, which was performed on the online platform of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). We also identified taxonomic markers from order to family level for single inoculant (M, C) and mixed inoculants (MF, CB, MFCB) at 30 days after the first inoculation. Briefly, based on the normalized relative abundance of each level, the Kruskal–Wallis (KW) rank-sum test was used to detect markers with significantly different abundances between the assigned taxa, and linear discriminant analysis (LDA) was performed to estimate the effect score of each marker (LDA threshold = 2). It emphasizes statistical significance, biological consistency and effect relevance, allowing researchers to identify differentially abundant features that are also consistent with biologically meaningful categories (Guerrero-Preston et al. 2016). High LDA scores reflect significantly higher abundance of certain taxa. To investigate the taxa–environment relationship, we performed redundancy analysis (RDA) with the soil bacterial community for all samples, the top 10 families, and environmental factors. Environmental factors for each sampling time were selected by variance inflation factor (VIF) analysis, which was used to judge the collinearity among different factors.

3. Results

Effects of microbial inoculants on dynamic growth of *Cyclocarya paliurus*.

The growth indices of *C. paliurus* were dynamically measured during the inoculation period (Baima) and transplantation period (Taizhou). In Baima, only treatment MFCB significantly increased the seedling height at 45 days after the first inoculation, while no significant improvement of ground diameter was found during this period. After the second inoculations, we observed improved plant height growth for treatments containing *B. megaterium* and *P. fluorescens,* i.e. MF and MFCB (Figure 4.2a, d), but no significant effects were found in other

treatments. In Taizhou, significant increases in plant height were observed in treatments MF and MFCB, but the differences in ground diameter between inoculated and non-inoculated seedlings were not significant. In terms of relative growth rate of height (RGRh) and ground diameter (RGRd), inoculations especially for MF, CB, and MFCB increased the RGRh and RGRd of *C. paliurus* in Baima, while a very limited impact of microbial inoculation was observed during the transplantation period (Figure 4.2c, f). Statistical results by Student's T-test indicated that the difference between each treatment in Baima and Taizhou was significant (P<0.05).

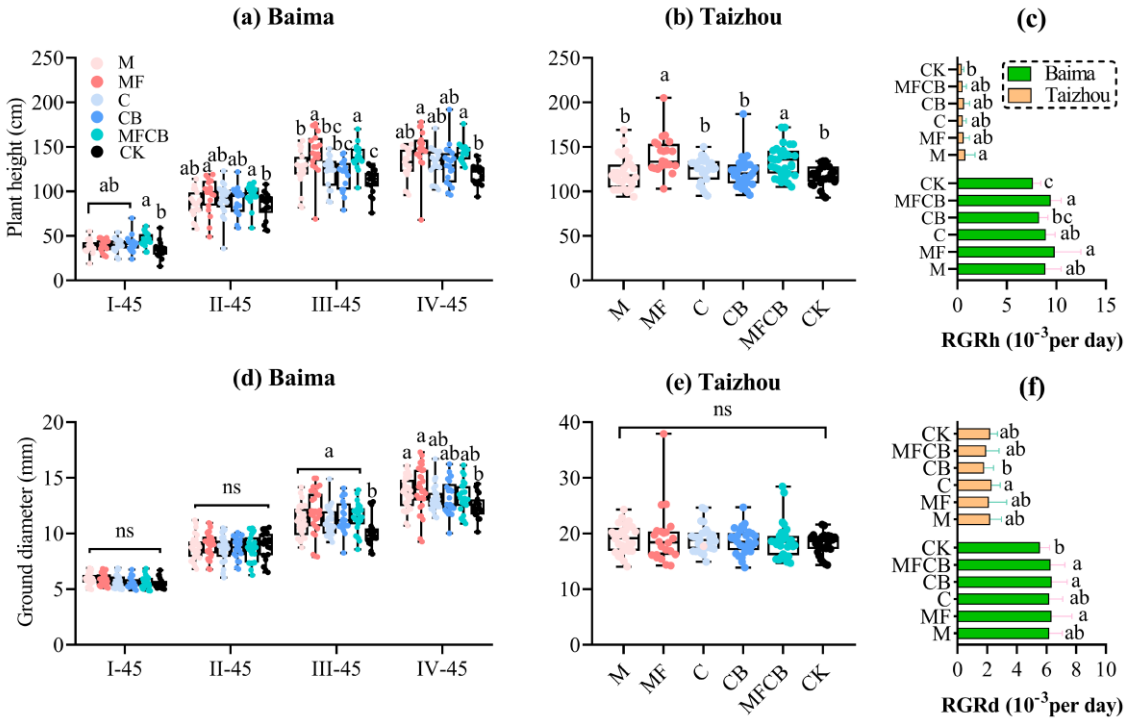


Figure 4.2 Dynamic growth of *C. paliurus* height (a) and ground diameter (d) in Baima (inoculation period), the final height (**b**) and ground diameter (**e**) of *C. paliurus* in Taizhou (transplantation period), and the relative growth rate of height (**c**) and ground diameter (**f**) in Baima and Taizhou. The sampling days were I-, II-, III-, and IV-45: 45 days after the first, second, third, and fourth inoculations, respectively. The treatments were M or C: single application of *Bacillus megaterium* or *Azotobacter chroococcum*, respectively; MF: dual application of *B. megaterium* and *Pseudomonas fluorescens*; CB: dual application of *A. chroococcum* and *Azospirillum brasilense*; MFCB: application of all four strains; CK: non-inoculation.

Soil biochemical properties

During the inoculation period, soils were collected at six time points (I-10, I-30, I-45, II-45, III-45, and IV-45) to determine soil biochemical properties. According to the two-way ANOVA results (Figure S1), the factors time

(varied from $P<0.0001$ to $P = 0.0339$) and inoculant type (varied from $P<0.0001$ to $P = 0.0687$) played key roles in explaining the variation of biochemical properties, but their interaction was not significant ($P>0.1$) for explaining the variations in soil pH and C/N ratio. After the first inoculation, soil available nutrients differed significantly between inoculated soils and the control during the first 10–90 days. However, the impacts of inoculation lessened over the period of 45–90 days, and the only significant differences were increases in SAP and SAN content in treatments MFCB and CB, respectively. Soil pH was lower in the first 10 days and the last 90 days compared with the control ($P<0.05$, Figure S1e and f). Inoculation time significantly influenced soil nitrogenase activity and acid phosphatase activity, but the patterns of change differed. Soil nitrogenase activity decreased at 45 days after the first inoculation (I-45) and recovered after the second and third inoculations. In contrast, phosphatase activity showed an increasing trend over the first 180 days, and a significant dependence of activity on inoculation time was also observed.

Bacterial diversity based upon 16S rRNA gene sequencing

After subsampling each of the total of 115 samples to an equal sequencing depth, a total of 10,978 OTUs at 97% identity were obtained, with a range of 1,952 to 2,932 OTUs per sample. According to the Good's coverage estimator (with an average of 97%) (Table S1), near-complete sampling of bacterial community diversity was obtained for all treatments. Compared with the OTU numbers at the time before inoculation (2607, data not shown), the observed OTUs significantly increased at I-10 and I-30, but little effect of treatment was observed (Table S2). Inoculation had no effect on the Shannon and Simpson indices after I-45, whereas the ACE (abundance-based coverage estimators) and Chao1 indices were significantly impacted by inoculation during the first 45 days. The effects of the different microbial consortia varied in the initial period; inoculation with four strains (MFCB) and two NFB (CB) increased Simpson values at I-10, whereas the Ace index was lower in the treatments with PSB (M and MF), as compared to the noninoculation treatment ($P<0.05$). According to the overall ANOVA test results (Table S3), sampling day significantly affected bacterial diversity and richness, but no significant effects of treatments or their interaction on bacterial diversity indices were observed across the entire study period.

Shifts of resident bacterial community composition under repeated microbial inoculations

The relative abundances of the top 11 phyla represented ~96% percent of the total communities (Figure S2). Most

of the bacterial sequences obtained from our experimental soils belonged to the phyla *Proteobacteria* (42-54%), *Bacteroidetes* (5-10%), *Actinobacteria* (5%) and *Acidobacteria* (5-21%); the remainder (16-20%) belonged to the phyla *Firmicutes*, *Chloroflexi*, *Gemmatimonadetes*, *Verrucomicrobia*, *Planctomycetes* and *Armatimonadetes*.

The bacterial community composition at the phylum level varied significantly across the different sampling times (180 days), with less pronounced effects of inoculation treatment (Table S4). However, there were significant differences in families between treatments, as shown in Figure 4.3 ($P < 0.05$). In response to periodic inoculations, the temporal variation of the top 50 families exhibited three distinct patterns with respect to time: resilience (a and c), antagonism (b), and synergism (d) (Figure 4.3). It should be noticed that the significant differences between treatments were mostly found within the first 45 days after the first inoculation (I-10, I-30, and I-45) in the pattern (a). In this period (0–45d), the relative abundances of families like *Pseudomonadaceae* and *Micrococcaceae* in all treatments, *Xanthomonadaceae* in MF and MFCB, and *Rhodanobacteraceae* in CB and MFCB significantly increased ($P<0.05$) compared to control. The family *Chitinophagaceae* decreased in the CB treatment, and *Anaerolineaceae* significantly decreased in all treatments ($P<0.05$). However, after 45 days, the bacterial community in pattern (a) exhibited resilience to the following disturbances, and no significant differences were found between inoculated and non-inoculated soils.

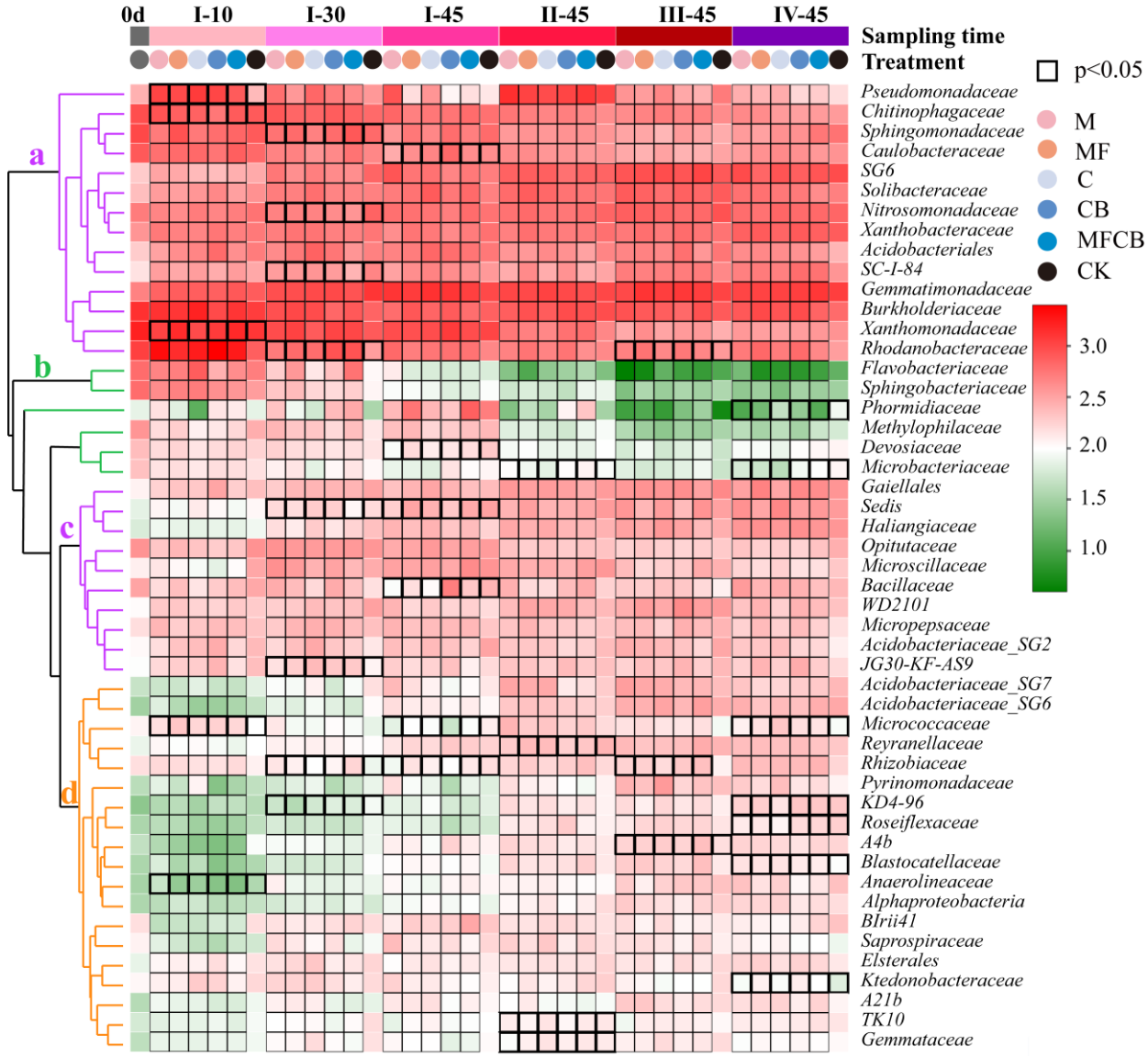


Figure 4.3 Heatmap of the bacterial community at the family level (top 50) under periodic inoculations over time. Black boxes indicate the statistical significance of differences between treatments at each time point. a, b, and c show different changing patterns of bacterial taxa across all sampling time points as clustered by based on abundance similarities between taxa. The sampling days were 0d: the day before microbial inoculation; I-10, I-30 and I-45: 10 days, 30 days, and 45 days after the first inoculation, respectively; II-, III-, and IV-45: 45 days after the second, third, and fourth inoculations, respectively. The treatments were M or C: single application of *Bacillus megaterium* or *Azotobacter chroococcum*, respectively; MF: dual application of *B. megaterium* and *Pseudomonas fluorescens*; CB: dual application of *A. chroococcum* and *Azospirillum brasilense*; MFCB: application of all four strains; CK: non-inoculation.

Effects of repeated microbial inoculations on overall bacterial community structure

PCoA ordination based on Bray-Curtis dissimilarities at the OTU level indicated the succession of the soil bacterial community over the course of the experiment (Figure 4.4a). In accordance with the result of community composition, the community changed significantly in the first 45 days (I-10, I-30, I-45) (PERAMONA, R^2 = 0.24, P = 0.001), but the community dissimilarities within the last three time points decreased. The pairwise correlations between different time points also indicated that the whole microbiome stabilized at last three time points (Figure S3).

To further examine the differences between inoculated and non-inoculated soil over time, typing analysis was conducted based on the Bray-Curtis dissimilarity in the PCoA plot (Figure 4.4b, c). At all seven time points (including 0d), three bacterial cluster types were found in inoculated soil, whereas only two bacterial cluster types were detected in the control (PERAMONA for five types, R^2 = 0.40, P = 0.01). Bar plots (Figure 4.4b, c) were used to depict the composition of these cluster types at each time point, showing that repeated inoculations altered the community succession as compared to non-inoculated treatment (Figure 4.4b). It took approximately 10–30 days for the bacterial community in non-inoculated soil to change from NonIno_0-10d (the community cluster in noninoculated samples during the first 10 days) to NonIno_30-180d (Figure 4.4b). The bacterial community in the inoculated soil also completed this change from Ino_0-10d (the cluster in the inoculated samples during the first 10 days) to Ino_30-45d, but after the second inoculation, Ino_30-45d was transformed into Ino_90-180d and remained stable thereafter (Figure 4.4c). To illustrate the dynamics of community composition and compare the differences between different cluster types, we identified the OTUs in different types and visualized the community succession on phylum level (Figure S4). The *Acidobacteria* phylum significantly increased in inoculated samples while stayed stable in non-inoculated soil. On the contrary, the Bacteroidetes phylum deceased over time in inoculated samples but increased in non-inoculated samples.

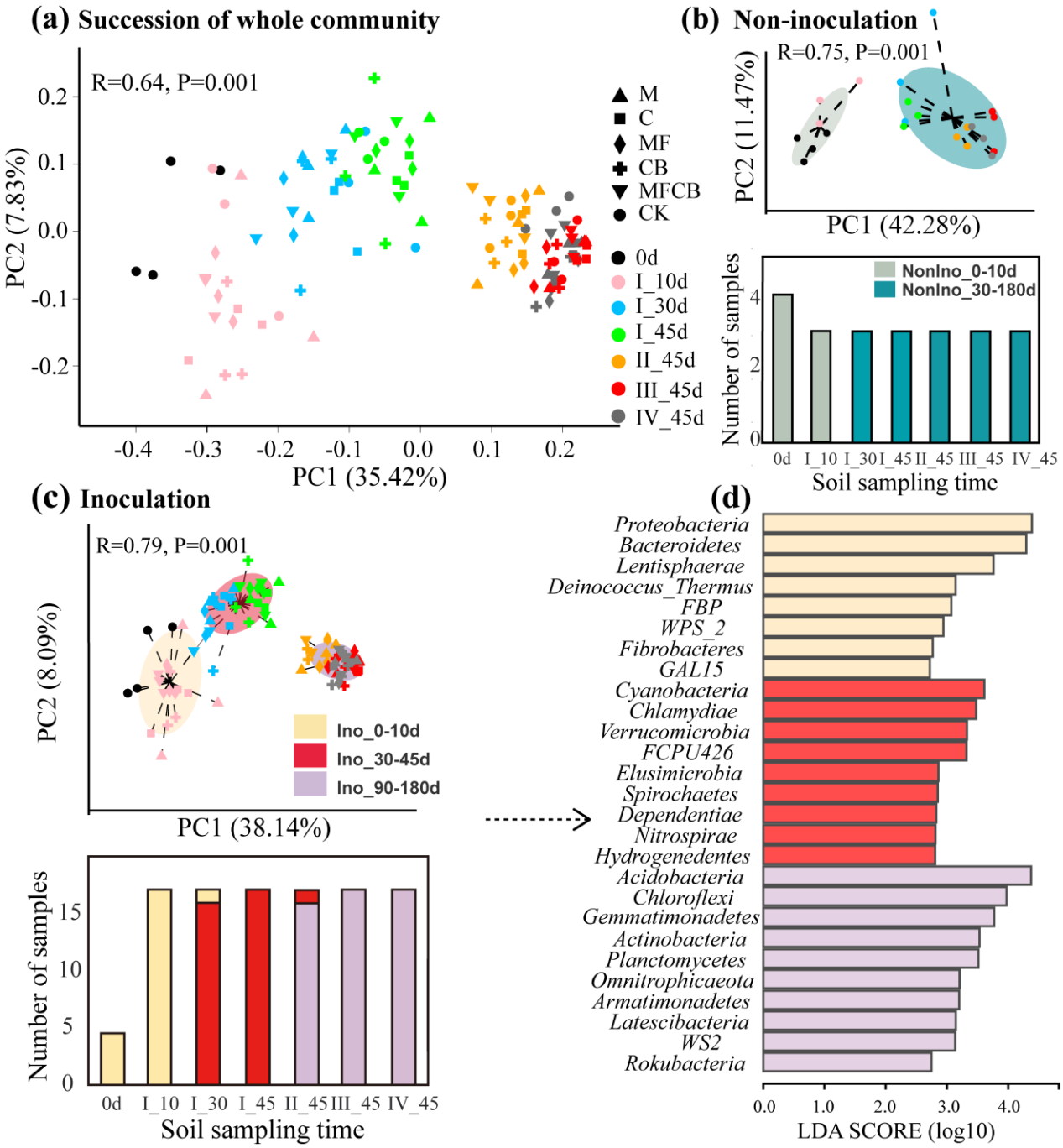


Figure 4.4 Temporal variation of bacterial community structure under different soil management. (**a**) Succession of the resident soil bacterial community as revealed by principal coordinates (PCo) of Bray–Curtis similarities. (**b**) Bacterial community clusters (PCoA plot) and their dominations (bar plot) in the succession of non-inoculated soils across all time points. NonIno_0-10d and NonIno_30-180d indicate two main clusters for non-inoculated samples as derived from community type analysis. (**c**) Bacterial community clusters and their dominations in the succession of inoculated soils across all time points. Ino_0-10d, Ino_30-45d, and Ino_90-180d indicate three main clusters for inoculated samples as derived from community type analysis. (**d**) Differences in phylum abundances among the three clusters found in the inoculated soils according to Linear discriminant analysis (LDA) score. The sampling days

were 0d: the day before inoculation; I-10, I-30 and I-45: 10 days, 30 days, and 45 days after the first inoculation, respectively; II-, III-, and IV-45: 45 days after the second, third, and fourth inoculations, respectively. The treatments were M or C: single application of *Bacillus megaterium* or *Azotobacter chroococcum*, respectively; MF: dual application of *B. megaterium* and *Pseudomonas fluorescens*; CB: dual application of *A. chroococcum* and *Azospirillum brasilense*; MFCB: application of all four strains; CK: non-inoculation.

Linear discriminant analysis (LDA) revealed differences in phylum abundances between the three cluster types (Ino_0-10d, Ino_30-45d, Ino_90-180d) found in inoculated soil (Figure 4.4d). The top 3 markers based on LDA scores were *Proteobacteria*, *Bacteroidetes* and *Lentisphaerae* for Ino_0-10d, *Cyanobacteria*, *Chlamydiae* and *Verrucomicrobia* for Ino_30-45d, and *Acidobacteria*, *Chloroflexi*, *Gemmatimonadetes* for Ino_90-180d. Soil properties (C, N, S, C/N, nitrate, pH and enzyme activity) were examined for their abilities to explain the bacterial community variation in inoculated soils (Figure 4.5). Among these factors, nitrate and acid phosphatase activity explained 46.1% and 42.3% of the bacterial community variation along axis 1, respectively, and soil pH explained the most variation along axis 2 (39.1%).

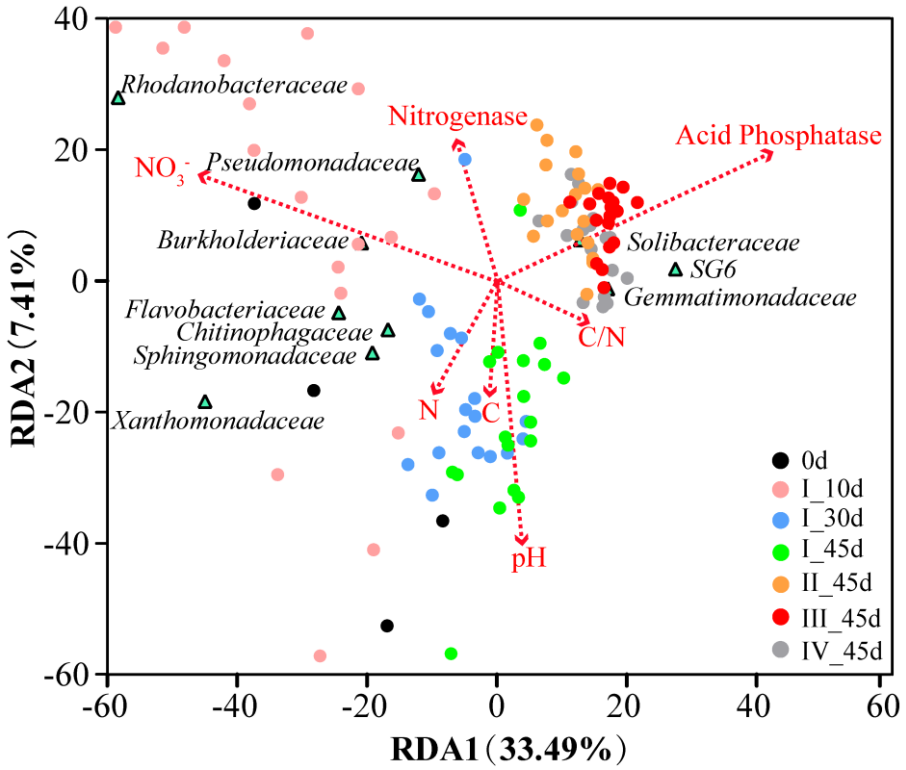


Figure 4.5 Redundancy analysis illustrating the effects of environmental factors on the succession of bacterial community and top 10 families across all treatments. The sampling days were 0d: the day before inoculation; I-10, I-30 and I-45: 10 days, 30 days, and 45 days after the first inoculation, respectively; II-, III-, and IV-45: 45 days after

the second, third, and fourth inoculations, respectively.

The route of community change is transiently modulated by single or mixed microbial inoculants

Since the dissimilarities in the bacterial community at II-45, III-45, and IV-45 were smaller than those in the first 45 days, we selected five time points in the first 45 days and last 45 days (Figure 4.6) to evaluate the different effects of the inoculant types on the soil resident bacterial community. Four cluster types (Types 1, 2, 3, 4) were obtained from these samples across these five time points, and the routes of community change from type 1 to type 4 differed according to treatments. The route was Type 1 – 2 – 4 for inoculation with mixed strains (MF, CB, MFCB) but Type 1 – 3 – 4 for the single-strain treatments (M, C) (Figure 4.6). In addition, across all five selected time points, a single – complex – single cluster pattern was observed (Figure 4.6, stacked column plot). These patterns suggest that the microbial inoculants modulated different subsets of the microbial community in soil for a short period, even though all inoculants ultimately resulted in similar clustering patterns.

Soil factors were analyzed to identify potential abiotic parameters affecting the succession of the resident microbial community over time (Figure 4.6). Inorganic N (nitrate and ammonium) and the activities of nitrogenase and acid phosphatase were the main factors driving the temporal variations of microbial community structure, whereas soil pH and C/N ratio, followed by nitrate, were the main factors explaining the difference between the single- and mixed-inoculant treatments at I-30. To explore the biological factors underlying the microbial community differences between the single- and mixed-inoculant treatments, we further compared taxonomic markers from the order to the family level at I-30 based on linear discriminant analysis effect size (LEfSe) (Figure S5). The top 3 markers in soils inoculated with mixed strains were *Xanthomonadales*, *Sphingomonadales*, and *Sphingomonadaceae*, whereas *Solibacteraceae*, *Solibacterales*, and *Thermoanaerobaculia* were the top 3 taxa in single-strain-inoculated soil.

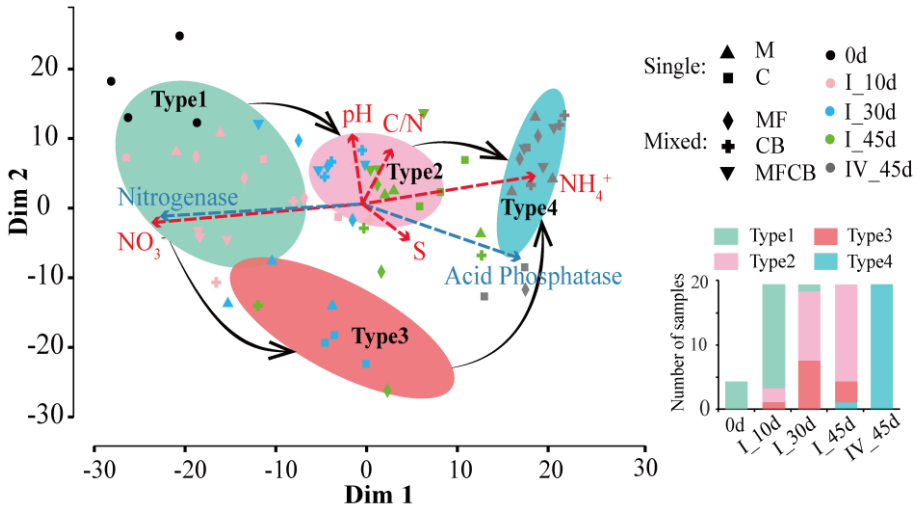


Figure 4.6 Typing analysis of the temporal variation of bacterial community structure under different inoculations at I-10, I-30, I-45, and IV-45. Different routes from type 1 to type 4 were identified: type 1 – 2 – 4 (for treatments MF, CB, and MFCB) or type 1 – 3 – 4 (for treatments M and C). The column diagram indicated a single – complex – single pattern of change the presence of the four cluster types at the five sampling time points. The sampling days were 0d: the day before inoculation; I-10, I-30 and I-45: 10 days, 30 days, and 45 days after the first inoculation, respectively; IV-45: 45 days after the second, third, and fourth inoculations, respectively. The treatments were M or C: single application of *Bacillus megaterium* or *Azotobacter chroococcum*, respectively; MF: dual application of *B. megaterium* and *Pseudomonas fluorescens*; CB: dual application of *A. chroococcum* and *Azospirillum brasilense*; MFCB: application of all four strains.

4. Discussion

The responses of plant growth and soil functioning to repeated inoculations

Soil beneficial microorganisms interact intimately with the roots of the host plant and affect the ecological adaptability of the plant to its environment. Nonetheless, these beneficial effects can be weakened by intensive land usage, thereby decreasing the plant's capacity to deal with biotic and abiotic stresses (Strigul and Kravchenko 2006). Consequently, we hypothesized the beneficial effects of microbial inoculants on soil nutrients and plant growth would increase with repeated applications. However, different from our hypothesis, we found that periodic inoculations mostly increased soil available nutrients during the first 10–90 days. Although the advantages of treatments MFCB and CB appeared to be pronounced at the last two sampling times, the benefits of inoculation generally decreased over time. This indicates the effects of microbial inoculants on bulk soil functioning were

transient rather than persistent during investigation. This is similar to previous study in which the inoculated strain caused no major changes on the rhizosphere community function (Krober et al. 2014). It should be noted the changes in the bulk soil might be different from that in the rhizosphere soil, and these changes can also in turn affect the microbial community (Kuramae et al. 2011). Nonetheless, in the present study, PCoA and community type analyses confirmed that the resident bacterial community in the bulk soil underwent shifts in the first 90 days but showed resilience to the subsequent inoculations in the last 90 days. This is in accordance with the observed variation of nitrogenase activity and soil inorganic nitrogen content. Given the resilience and resistance of the resident microbiome (Griffiths and Philippot 2013), we speculate that this decrease could be due to changes in, or stability of, the soil microbial community. In addition, the decrease in soil nutrient content at last time points may also be due to seasonal variation and nutrient uptake by plants. Our previous study confirmed that these microbial inoculants enhanced nutrient uptake and stimulated plant growth and biomass accumulation after whole inoculation procedures (Wang et al. 2019c).

Even though introduced microbial inoculants sometimes cannot compete efficiently with native microbial communities in soil, they stimulate root growth and modify plant metabolism at very early stages and might generate lasting effects on the root system and associated microbial communities (Bashan 1999). In the present study, microbial inoculations significantly promoted *C. paliurus* growth and reshaped root morphological traits (more fine roots and lateral roots in the inoculated seedlings, data not shown) compared to non-inoculated seedlings after the inoculation period in Baima. However, the growth-promoting effect was highly variable across time and inoculums and not maintained when the seedlings were transplanted to Taizhou. The subsequent growth-promoting effects of microbial inoculants on plants might be compromised due to the ceased inoculation, thus presenting the importance of continuous microbial inoculation when transplanting and establishing plantation in a different site. Another reason could be that the change of soil environment, because plants exhibit less reliance on the soil beneficial microbes when experiencing a normal/rich level of nutrient environment, thus benefit less from the previous inoculation (Altieri and Nicholls 2003). Even though we cannot precisely track the establishment of introduced strains in a different site, but the results proved the benefits from inoculation could decrease without subsequent applications.

Inoculation times and type affect the composition and succession of the resident bacterial community

Both natural and anthropogenic microbial invasions frequently start with a dominating microbial population and

leave a footprint on the native soil microbiome, even though the introduced populations may decrease at last (Saison et al. 2006, Mallon et al. 2018). With the increasing demand for bio-fertilizers in agroecosystems, the question of whether repeated application of bio-fertilizer (such as beneficial microbial inoculants) influences the resident soil community warrants investigation. In addition, with regard to the introduction of plant growth promoting rhizobacteria (PGPR), previous studies (Zhang et al. 2019, Zhuang et al. 2020) have attempted to evaluated the impacts on microbial community in the rhizosphere while less is focusing on the changes of bulk soil community. To address these questions, we evaluated to what extent and how long the repeated applications of inoculants (not native) impacted the dynamics of the resident bacterial community in the bulk soil. In response to repeated inoculations, three patterns (fold increased, fold decreased, and resilience) of shifts in bacterial composition were observed, 57% of the significant variation among treatments occurred during the first 45 days. Changes in soil nutrients were consistent with these shifts. Furthermore, microbial inoculants may alter resident community composition by causing resource competition, synergistic effect, and antagonistic effect (Mawarda et al. 2020). In the present study, the relative abundances of families like *Xanthomonadaceae* significantly increased in the treatments with PSB, suggesting that the introduction of PSB facilitated specific resident populations, which is in accordance with previous study (Kuramae et al. 2011). In contrast, *Chitinophagaceae* and *Rhodanobacteraceae* significantly decreased in soil inoculated with NFB. These declines in the abundance of some taxa after the initial disturbance due to microbial inoculation may be a result of competition for similar preferred niches and available resources in the soil (Krause et al. 2014, Mallon et al. 2018).

The resident soil bacterial community exhibited a high level of resilience, but not resistance, to the microbial disturbance caused by periodic inoculations. The initial inoculation disturbed the stability of the resident microbiome, which was as a result more susceptible to subsequent inoculation disturbances. This is in line with above discussion that the effects of such amendments on below-and above ground are both transient. PCoA, community type and pairwise correlation analyses confirmed that the dissimilarity between the communities decreased in the last 90 days. This suggests resilience of the resident microbiome upon repeated inoculation disturbances, similar to other reports of resilience within soil microbial communities (Allison and Martiny 2008, Lourenco et al. 2018). Surprisingly, in the present study, the second inoculation still left a footprint on the resident community, resulting in an increase in the number of cluster types in the inoculated soils (Ino_0-10d, Ino_30-45d , Ino_90-180d) compared with the control (NonIno_0-10d, NonIno_30-180d). This finding also confirms the previously proposed hypothesis that a second disturbance by the same invader could persist longer or even

naturalize into the community (Mallon et al. 2018). It should be mentioned that we did not use specific primers to track the persistence of inoculated strains in soil; however, 16S rRNA gene sequencing showed that the introduction of microbial inoculants altered the seasonal succession of the resident community. The unexpectedly stronger impact of soil management over temporal effects on the resident community is supported by previous observations in different agricultural systems (Hartmann et al. 2015, Fu et al. 2017), but this study revealed the relationship between repeated inoculations and the native microbiome. Although this work provided the detailed information about how inoculation period and type affect the resident microbes, the future study should consider to set a unique control which receives only one dose at first and sampled at the end of experiment, to further compare the influences of repeated inoculation compared to one-off inoculation. Furthermore, insignificant (Piromyou et al. 2013) and significant effects (Liu et al. 2018a) were both observed on the native microbial community structure in the rhizosphere soils after PGPR inoculation. Hence, it would be interesting that the future studies can compare the differences of community succession in bulk soil and rhizosphere soil.

Underlying factors in shaping resident microbiome during the application of microbial inoculants

The changes in soil chemical factors due to beneficial microbial inoculation, such as nitrate and pH, were the dominant factors explaining the succession of the resident community over time. Kuramae et al. (Kuramae et al. 2010) also reported that soil pH significantly altered the trajectory of microbial secondary succession. Confirming this result, after the first and the fourth inoculations, soil pH in inoculated treatments significantly differed from that in non-inoculated soil. As the PSB possess the ability of producing organic acid during the decomposition of soil organic matters, which is associated with the release of P from mineral-bound complexes such as $AlPO_4$ and $FePO_4$, thus leading to a decrease of soil pH and change the related nutrient contents (Orhan et al. 2006). On the other hand, NFB are able to increase the contents of ammonium and consequently improve the nitrites with the help of nitrifying bacteria. In the present study, the contents of inorganic N after the first inoculation were significantly increased compared to control. To evaluate the potential impacts of the growth medium on the change of soil properties and plant performance, we confirmed that the addition of bacterial growth medium exhibited no significant impacts on plant growth, biomass, and nutrient acquisition and showed very limited influence on soil available nutrients (Wang et al. 2019c). However, it cannot be ruled out that other factors, not assessed in this study, might be driving this seasonal variation.

For the identified taxonomic markers for each cluster in the inoculated soils, phyla *Proteobacteria* and

Bacteroidetes generally have copiotrophic strategies with rapid growth responses to resource availability (Fierer et al. 2007). In this study, these phyla were enhanced during the first 45 days after inoculation, which is also the period for the rapid change of soil nutrients. *Cyanobacteria* are emerging beneficial microorganisms with the ability to control nitrogen deficiency and sensitivity to fertilization (Song et al. 2005, Singh et al. 2016), whereas *Chlamydiae* and *Verrucomicrobia* are sensitive to soil moisture and time (seasonal variation) (Buckley and Schmidt 2001, Wagner and Horn 2006). These phyla were significantly more abundant in cluster Ino_30-45d than in the other cluster types, indicating contributions of both microbial inoculation and seasonal variation. The presence of *Acidobacteria* in cluster Ino_90-180d is likely attributable to the low soil pH at the last sampling time compared with the control, which seems to favor this bacterial phylum (Kielak et al. 2016). The phyla *Chloroflexi* and *Gemmatimonadetes* are widely known to be enriched in dry season soil (DeBruyn et al. 2011, Lacerda-Júnior et al. 2019). Overall, the formation of different cluster types is likely attributable to both seasonal variation and changes in soil biochemical properties caused by periodic inoculations.

Mixed inoculants of different strains have been widely developed and evaluated for their great potential in enhancing plant growth and soil nutrients (Juge et al. 2012, Yu et al. 2012, Hungria et al. 2013). In this study, plant growth exhibited a strong preference for mixed inoculants MFCB, which presented the highest growth of height and ground diameter during the whole inoculation period. Dual inoculant such as MF also showed significant advantages than single inoculant M in improving soil enzyme activities at certain time points. It has been proposed that co-inoculation permits synergistic interactions that stimulate physical or biochemical activities and simultaneously improve microbial viability (Yu et al. 2012), thus bringing more interaction with soil and host plant such as the production of enzymes and organic acid. On the other hand, co-inoculation may leave a different footprint on the resident microbiome compared to single inoculation, because more ecological niches would be required for mixed inoculants than when these organisms were used alone (Paerl and Pinckney 1996, Yu et al. 2012, Wei et al. 2018a). In addition, the nature of such differences could also due to the feedback of changed soil environments and plant performance. In the present study, different inoculants (single/mixed) transiently modulated the variation of the resident community at 30 days after the first inoculation. Soil pH and the C/N ratio were the main factors underlying this impact, followed by nitrate. Confirming this result, soil C/N at I-30 was higher in single inoculants than in mixed inoculants. However, the difference in pH between the single- and mixed-inoculant treatments was not significant. Hence, other environmental factors that were not assessed in this study could be driving these differences. For the biotic factors, bacterial taxa like *Solibacteraceae*, *Solibacterales*, and

Thermoanaerobaculia (all belonging to the phylum *Acidobacteria*) were identified as markers for the single treatments based on LDA score. The abundance of the phylum *Acidobacteria* is closely related to soil pH and resources such as total nitrogen and nitrate (Navarrete et al. 2013, Kielak et al. 2016, Kuramae and de Assis Costa 2019), being consistent with the soil factors discussed above. It should be noted that succession difference of resident community derived from single and mixed inoculants was only observed for a short period, the resident community established and behaved similarly at last.

In conclusion, repeated inoculations did not ideally improve the benefits from microbial inoculants, and the beneficial effects on plant growth were not maintained after transplanting to a different site. Consequently, the necessity of repeated microbial inoculations should be reconsidered. The resident bacterial community in bulk soil exhibited traits of resilience, but not resistance, to repeated inoculation. This study revealed that the changes in the resident community mostly reflected the initial disturbance of inoculant addition and partially explained the variations in soil nutrients and subsequent plant growth. The responses of bacterial taxa in the soil to microbial inoculants depended on the inoculant types (PSB or NFB) and taxa clusters. In response to periodically introduced microbes, resilient changing pattern included the main taxa of resident microbiome. Inoculation and non-inoculation significantly differed during the succession of community and resulted in different cluster types and composition shifts, thus providing a new insight into understanding the interactions between resident microbes and intruders. Soil pH and nitrate were the main factors explaining the succession of the resident community, leading to the development of three cluster types over time. The single and mixed inoculants briefly modulated the variation of the resident community in association with soil pH and the C/N ratio. However, over time, bacterial communities established and showed high level of resilience.

Supplementary material

Table S1 Sample ID, sampling day, treatment, inoculant type, number and length of 16S rRNA gene sequences used in this study

Sample_ID	Sampling_day (d)	Treatment	Inoculant_type	Reads per sample	Mean_length	Coverage[1]
A1	0	0d	-	48921	375.67	0.9686
A2				39906	375.82	0.9685
A3				47382	375.52	0.9719
A4				49994	375.70	0.9734
B1_1	I_10	M	PSB[2]	43345	376.08	0.9673
B1_2				52355	375.58	0.9692
B1_3				48932	376.01	0.9707
B2_1		C	NFB[3]	56378	375.66	0.9742
B2_2				49395	375.70	0.9702
B2_3				53023	376.03	0.9674
B3_1		MF	PSB	52930	376.16	0.9705
B3_2				56317	375.65	0.9718
B3_3				56056	375.89	0.9680
B4_1		CB	NFB	45021	376.07	0.9752
B4_2				54466	376.16	0.9721
B4_3				71557	375.94	0.9730
B5_1		MFCB	PSB+NFB[4]	58807	376.01	0.9728
B5_2				54509	375.86	0.9740
B5_3				54591	375.88	0.9734
B6_1		CK	CK	53211	376.09	0.9737
B6_2				55118	375.68	0.9731
B6_3				73585	375.75	0.9728
C1_1	I_30	M	PSB	60018	376.04	0.9722
C1_2				63039	375.85	0.9694
C1_3				66388	376.00	0.9725
C2_1		C	NFB	64197	376.05	0.9734
C2_2				57061	375.98	0.9715
C2_3				64206	376.10	0.9744
C3_1		MF	PSB	48211	376.02	0.9675
C3_2				47685	375.79	0.9633
C3_3				48980	375.97	0.9627
C4_1		CB	NFB	53151	376.13	0.9650
C4_2				45751	376.02	0.9656
C4_3				54885	375.96	0.9635
C5_1		MFCB	PSB+NFB	44651	375.70	0.9678
C5_2				48346	376.02	0.9672

C5_3				52503	376.12	0.9623
C6_1				48299	375.95	0.9635
C6_2		CK	CK	46895	376.19	0.9680
C6_3				58524	376.48	0.9814
D1_1				39946	376.35	0.9711
D1_2		M	PSB	47086	376.02	0.9644
D1_3				48179	376.20	0.9642
D2_1				57055	376.20	0.9662
D2_2		C	NFB	52573	376.10	0.9646
D2_3				46776	376.17	0.9622
D3_1				55436	376.17	0.9668
D3_2		MF	PSB	73796	376.11	0.9743
D3_3				60691	376.20	0.9684
D4_1	I_45			62610	376.09	0.9714
D4_2		CB	NFB	62047	376.20	0.9697
D4_3				73616	376.18	0.9748
D5_1				45888	376.15	0.9608
D5_2		MFCB	PSB+NFB	54539	376.16	0.9662
D5_3				53492	376.05	0.9635
D6_1				53381	376.09	0.9632
D6_2		CK	CK	56591	376.11	0.9659
D6_3				46532	376.09	0.9600
F1_1				59922	376.34	0.9664
F1_2		M	PSB	50301	376.26	0.9626
F1_3				49140	376.23	0.9577
F2_1				57647	376.31	0.9633
F2_2		C	NFB	49042	376.26	0.9610
F2_3				59258	376.29	0.9716
F3_1				52737	376.27	0.9633
F3_2		MF	PSB	55177	376.16	0.9657
F3_3				58206	376.25	0.9660
F4_1	II_45			51058	376.47	0.9637
F4_2		CB	NFB	49320	376.29	0.9662
F4_3				55687	376.19	0.9638
F5_1				54793	376.34	0.9608
F5_2		MFCB	PSB+NFB	43272	376.21	0.9532
F5_3				50481	376.16	0.9621
F6_1				38403	376.23	0.9469
F6_2		CK	CK	44836	376.29	0.9604
F6_3				44737	376.15	0.9580
G1_1	III_45	M	PSB	45469	376.35	0.9580

G1_2				53225	376.34	0.9625
G1_3				58104	376.26	0.9645
G2_1				58139	376.37	0.9666
G2_2		C	NFB	40303	376.48	0.9469
G2_3				52116	376.40	0.9608
G3_1				58100	376.46	0.9655
G3_2		MF	PSB	62086	376.29	0.9681
G3_3				61197	376.44	0.9675
G4_1				64088	376.44	0.9670
G4_2		CB	NFB	36281	376.41	0.9509
G4_3				45265	376.32	0.9556
G5_1				47905	376.34	0.9641
G5_2		MFCB	PSB+NFB	55625	376.34	0.9643
G5_3				51043	376.38	0.9644
G6_1				68323	376.34	0.9697
G6_2		CK	CK	54653	376.38	0.9670
G6_3				51328	376.24	0.9622
E1_1				55370	376.33	0.9641
E1_2		M	PSB	49742	376.28	0.9597
E1_3				52131	376.34	0.9623
E2_1				62898	376.35	0.9706
E2_2		C	NFB	67019	376.42	0.9715
E2_3				49719	376.32	0.9615
E3_1				50326	376.37	0.9631
E3_2		MF	PSB	69329	376.35	0.9706
E3_3	IV_45			51928	376.33	0.9609
E4_1				55319	376.39	0.9646
E4_2		CB	NFB	45696	376.39	0.9587
E4_3				48518	376.43	0.9600
E5_1				48462	376.41	0.9613
E5_2		MFCB	PSB+NFB	52052	376.35	0.9632
E5_3				51562	376.45	0.9620
E6_1				47230	376.43	0.9580
E6_2		CK	CK	51153	376.46	0.9593
E6_3				50983	376.31	0.9615

After quality filtering, 112 samples yielded a total of 5,985,527 16S rRNA gene sequences with an average of 53,442 reads per sample. The length of the trimmed sequences ranged between 360 bp and 400 bp. The treatments are: 0d: soil sampled before bio-fertilization; M or C: single application of *Bacillus megaterium* or *Azotobacter chroococcum*; MF: dual application with *B. megaterium* and *Pseudomonas fluorescens*; CB: dual application with *A. chroococcum* and *Azospirillum brasilence*; MFCB: application with four strains; CK: non-inoculation. The sampling day are: I-10, I-30 and I-45: 10 days, 30 days, and 45 days after the first bio-fertilization, respectively. II-, III-, and IV-45: 45 days after the second, third, and fourth bio-fertilization, respectively.

[1] Coverage: Good's non-parametric coverage estimator.

[2] PSB: phosphate solubilizing bacteria

[3] NFB: nitrogen fixing bacteria

[4] PSB+NFB: combined with PSB and NFB

Table S2 Effects of sampling time and treatments on the OTUs, diversity and richness.

Treatments	Sampling time					
	I-10	I-30	I-45	II-45	III-45	IV-45
			OTUs			
	B	A	A	A	A	A
M	2874	3673.3	3279.7ab	3097.7	2953	3190.7
MF	3027.7	3304	3643.7a	3050.7	3113	3220
C	2925	3807	3226ab	3326.3	2933.3	3355.7
CB	3016	3221.7	3257.3b	3142.7	2948.3	3133.3
MFCB	3100.7	3190	3171ab	3086.7	2916	3155.7
CK	3275.3	3472.7	3296.7ab	3055.7	3209.7	3209.7
			Shannon			
M	6.02B	6.69bA	6.65abA	6.58A	6.72A	6.78A
MF	6.25B	6.69abAB	6.76abA	6.6AB	6.72AB	6.77A
C	6.04B	6.79aA	6.74aA	6.77A	6.72A	6.76A
CB	6.07B	6.58abA	6.51bAB	6.62A	6.79A	6.76A
MFCB	6.13B	6.64abA	6.63bA	6.62A	6.73A	6.79A
CK	6.42B	6.77abAB	6.7aAB	6.73AB	6.75AB	6.84A
			Simpson			
M	0.0225abA	0.004B	0.0045B	0.0078AB	0.0033B	0.0029B
MF	0.0088a	0.0035	0.0042	0.0063	0.0032	0.0029
C	0.0133ab	0.0033	0.0031	0.0045	0.0034	0.0031
CB	0.0159a	0.0052	0.0049	0.0055	0.0027	0.003
MFCB	0.0123a	0.0044	0.0047	0.0092	0.0031	0.0028
CK	0.0057b	0.0031	0.0038	0.0057	0.0036	0.0027
			Ace			
M	4406.3b	5069.1ab	4596.6	4345.9	4156.9b	4539.7
MF	4339.7b	5274.3ab	5037.6	4271.3	4327.4a	4511.9
C	4674.9a	5215.6a	5098.4	4512.1	4193.6ab	4645.3
CB	4290.2b	4621.2ab	4892.5	4394.5	4184.5ab	4678.3
MFCB	4663.8ab	4514.1b	5027.8	4376.9	4023.5b	4415.9
CK	5186.2a	4723.4ab	4663.4	4343.6	4420.7ab	4549.6
			Chao1			
M	4151.6b	5118a	4662.5ab	4340.8	4129a	4534.5
MF	4398.9aB	4822.1bAB	5072.4aA	4224.5AB	4246.8aAB	4620.5AB
C	4259.3abB	5162.9aA	4698.8abAB	4451AB	4135.9abAB	4623.3A
CB	4280.0abB	4628.3bA	4668.6abAB	4368AB	4090.3abAB	4452.7A

MFCB	4508.9a	4545.2ab	4645.4b	4317.3	3928.1b	4432
CK	4814.6a	4693.9ab	4662.5b	4301.6	4348.7a	4570

Values within the same column followed by the different lowercases indicate significant difference ($P < 0.05$) among treatments, capital letters indicate significant difference ($P < 0.05$) among sampling time by Tukey's test. The treatments were M or C: single application of *Bacillus megaterium* or *Azotobacter chroococcum*, respectively; MF: dual application of *B. megaterium* and *Pseudomonas fluorescens*; CB: dual application of *A. chroococcum* and *Azospirillum brasilense*; MFCB: application of all four strains; CK: non-inoculated treatment.

Table S3 Overall ANOVA test results for the whole treatments and sampling time

ANOVA test	**Shannon**	**Simpson**	**Ace**	**Chao**
Treatment	ns	ns	ns	*
Day	****	****	**	****
Treatment × day	ns	ns	ns	ns

"ns" means no significance. Significant difference: *p≤0.05; **p≤0.01; ***p≤0.001. **** p≤ 0.0001.

Table S4 Significance between all sampling time for each treatment and significance between all treatments for each sampling time by Kruskal-Wallis H test.

Phyla	**Significance between six sampling time for each treatment**					
	M	**MF**	**C**	**CB**	**MFCB**	**CK**
Proteobacteria		**	*		**	
Acidobacteria	**	***	***	***	***	***
Bacteroidetes	*	**	***	**	**	**
Actinobacteria		*				
Chloroflexi	**	***	**	***	**	**
Gemmatimonadetes	*	**	**	**	**	*
Planctomycetes	*	*	***	**		**
Cyanobacteria			*	*	**	*
Firmicutes	*		*			
Verrucomicrobia	***	*			*	**
Armatimonadetes	*	***	**	***	**	**
Phyla	**Significance between six treatments for each sampling time**					
	I-10	**I-30**	**I-45**	**II-45**	**III-45**	**IV-45**
Proteobacteria						
Acidobacteria		C^+		C^+		
Bacteroidetes						
Actinobacteria						
Chloroflexi						
Gemmatimonadetes						
Planctomycetes					M^-, MF^-	M^-, C^-
Cyanobacteria				CK^-		
Firmicutes						

	Verrucomicrobia	
	Armatimonadetes	MF^+

"ns" means no significance. Significant difference: *p≤0.05; **p≤0.01; ***p≤0.001.

C^+ : The abundance of *Acidobacteria* was increased in treatment C but decreased in treatments by comparing to CK

M^-, MF^-, C^- :The abundance of *Planctomycetes* was decreased in treatment M, MF and C by comparing to CK

CK^- : The abundance of *Cyanobacteria* was decreased in CK by comparing to other treatments

MF^+: The abundance of *Armatimonadetes* was decreased in treatment MF by comparing to CK;

The treatments are: M or C: single application of *Bacillus megaterium* or *Azotobacter chroococcum*; MF: dual application with *B. megaterium* and *Pseudomonas fluorescens*; CB: dual application with *A. chroococcum* and *Azospirillum brasilence*; MFCB: application with four strains; CK: non-inoculation. The sampling day are: I-10, I-30 and I-45: 10 days, 30 days, and 45 days after the first bio-fertilization, respectively. II-, III-, and IV-45: 45 days after the second, third, and fourth bio-fertilization, respectively.

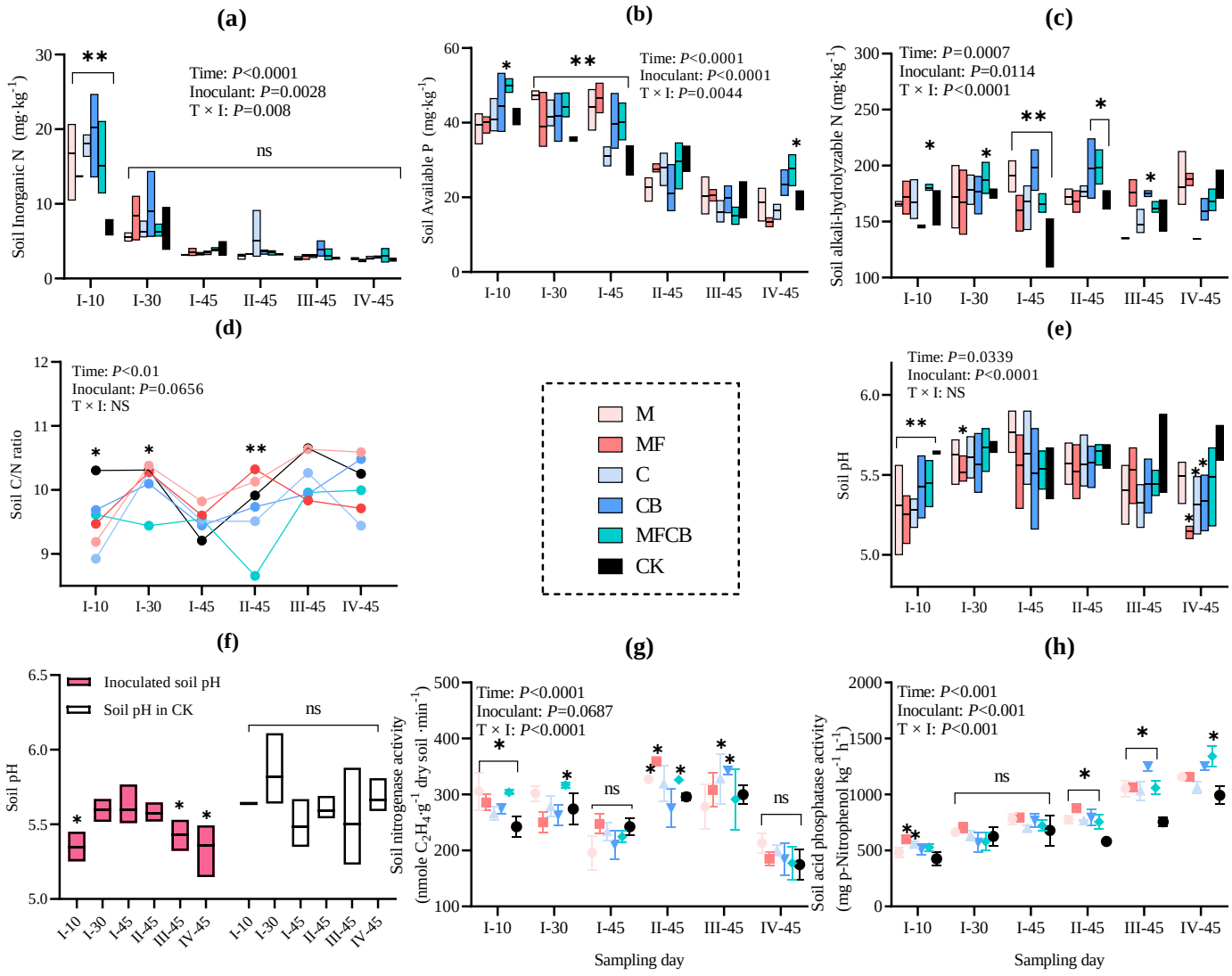


Figure S1 Responses of (a) soil inorganic N, (b) soil available P, (c) soil alkali-hydrolyzable N, (d) C/N ratio, (e) soil pH, (f) average soil pH under inoculation and control, (g) soil nitrogenase activity, and (h) soil acid phosphate activity to inoculant types and sampling time. “ns” means no significance. Significant difference: *p≤0.05; ** p≤ 0.01. The treatments are: M or C: single application of *Bacillus megaterium* or *Azotobacter chroococcum*; MF: dual application with *B. megaterium* and *Pseudomonas fluorescens*; CB: dual application with *A. chroococcum* and *Azospirillum brasilence*; MFCB: application with four strains; CK: non-inoculation. The sampling day are: I-10, I-30 and I-45: 10 days, 30 days, and 45 days after the first bio-fertilization, respectively. II-, III-, and IV-45: 45 days after the second, third, and fourth bio-fertilization, respectively.

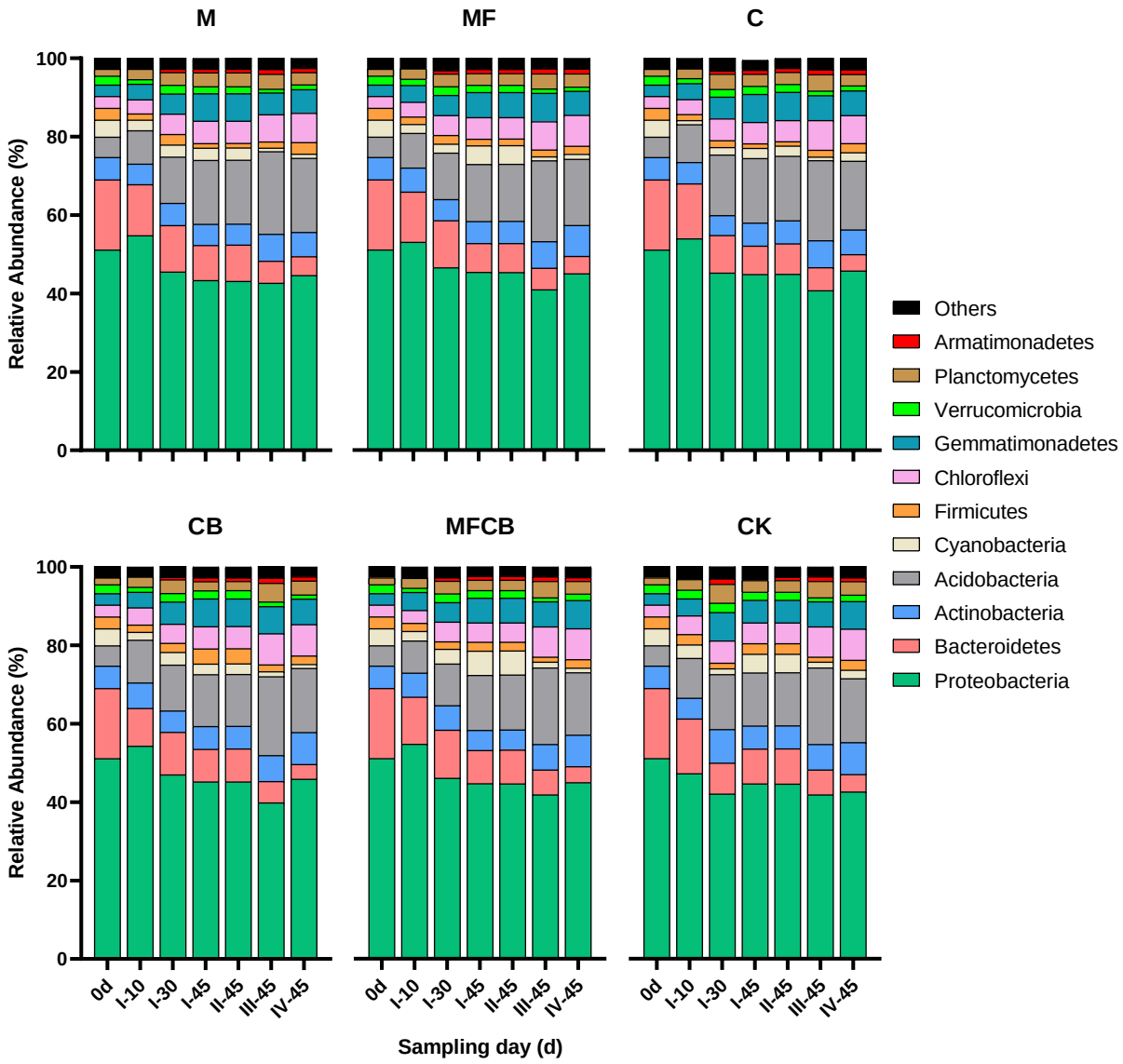


Figure S2 Relative abundance (%) of soil microbial phyla in soils under different treatments. The value of each bacterial group percentage is the mean of soil samples collected from three different replicates. The treatments are: M or C: single application of Bacillus megaterium or Azotobacter chroococcum; MF: dual application with B. megaterium and Pseudomonas fluorescens; CB: dual application with A. chroococcum and Azospirillum brasilence; MFCB: application with four strains; CK: non-inoculation. The sampling day are: 0d: the soil samples before the bio-fertilization; I-10, I-30 and I-45: 10 days, 30 days, and 45 days after the first bio-fertilization, respectively. II-, III-, and IV-45: 45 days after the second, third, and fourth bio-fertilization, respectively

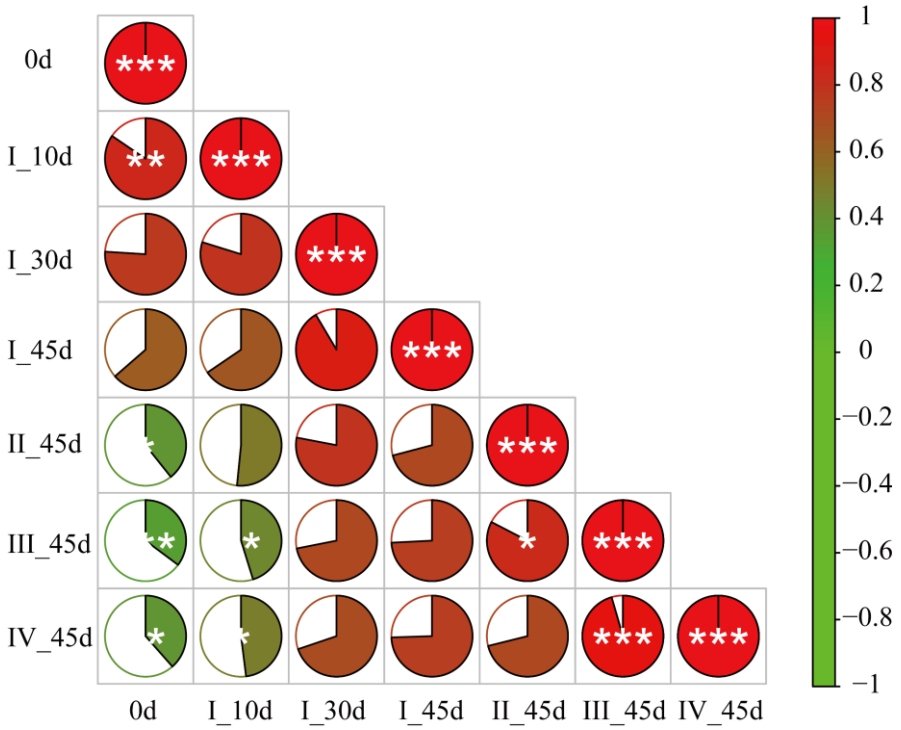


Figure S3 Pairwise correlations of whole soil microbiome between time points. The sampling day are: 0d: the soil samples before the bio-fertilization; I-10, I-30 and I-45: 10 days, 30 days, and 45 days after the first bio-fertilization, respectively. II-, III-, and IV-45: 45 days after the second, third, and fourth bio-fertilization, respectively. Significant difference: *p≤0.05; ** p≤ 0.01, *** p≤ 0.001.

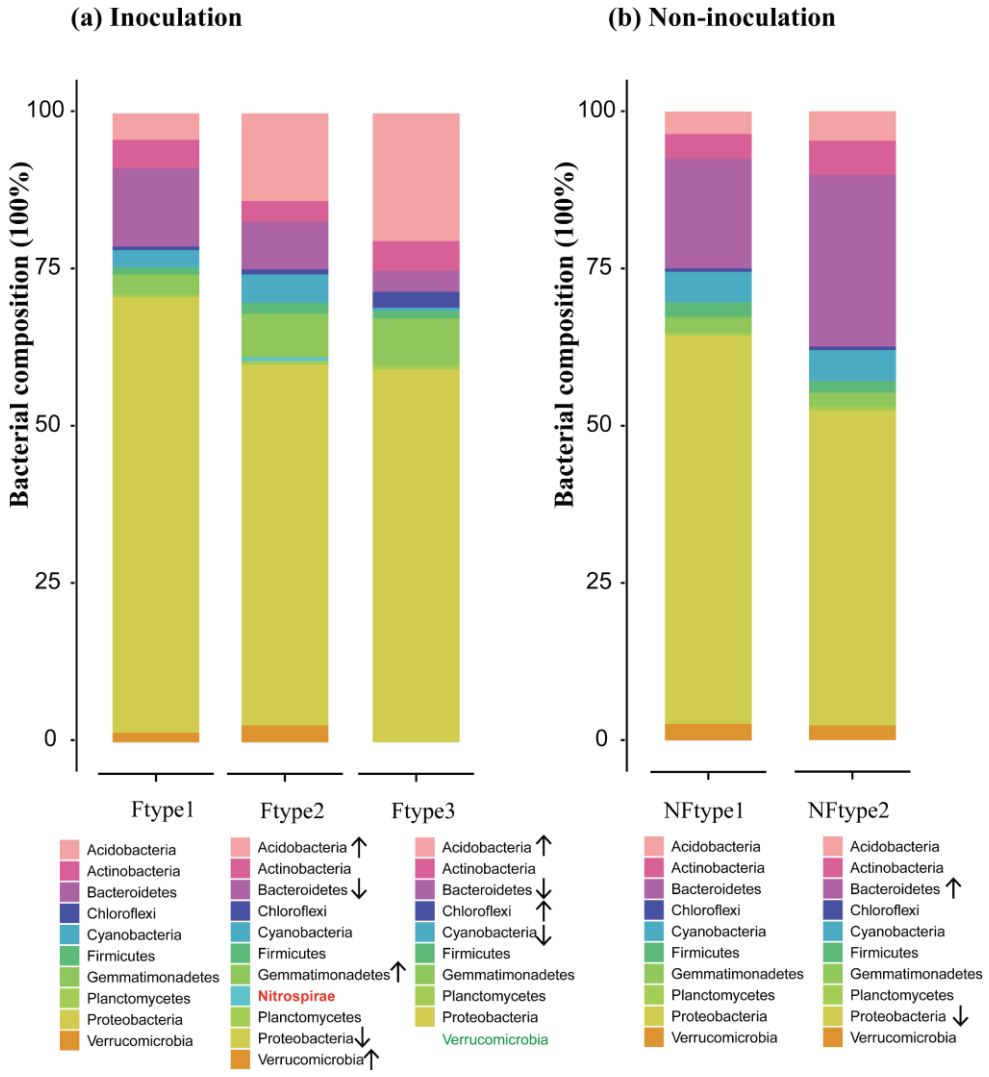


Figure S4 Community composition of different cluster types in (a) inoculated and (b) non-inoculated soils. "↑" means the phylum significantly increased compared to that in the former cluster type, "↓"means the phylum significantly decreased compared to that in the former cluster type. The phylum name in red means a new phylum was detected compared to the first type; the phylum name in green means the phylum disappeared compared to the first type.

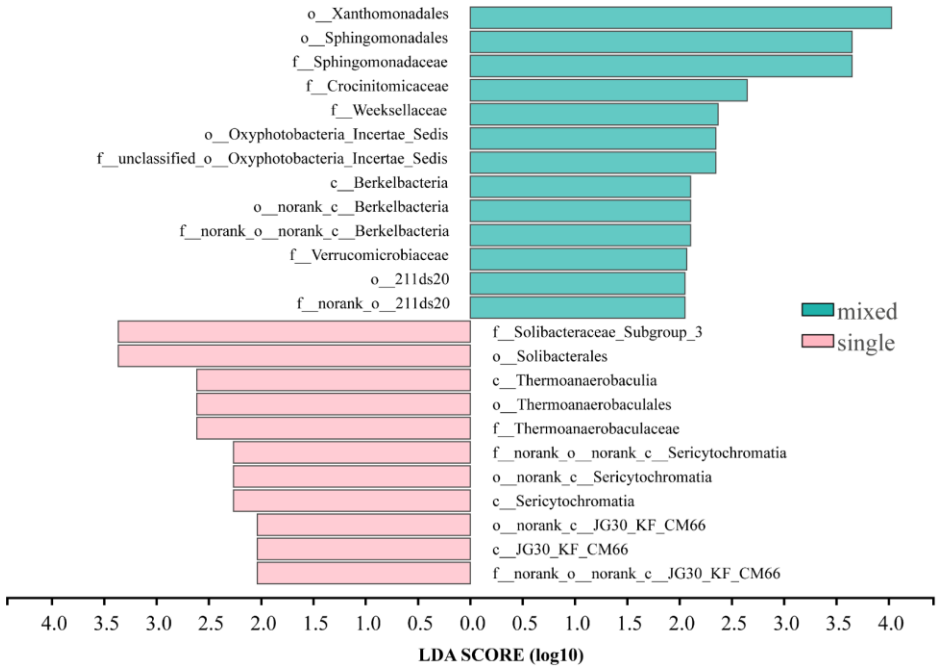


Figure S5 Linear discriminant analysis of abundances from order to family level between single inoculant (M, C) and mixed inoculants (MF, CB, MFCB) at 30 days after the first PGPR inoculation.

Chapter 5 Microbial inoculants modulate morphological traits and bioactive compounds of *Cyclocarya paliurus* (Batal.) Iljinskaja under degraded field condition

Adapted from: Wang Z, Xu Z, Chen Z, et al. Microbial inoculants modulate growth traits, nutrients acquisition and bioactive compounds accumulation of *Cyclocarya paliurus* (Batal.) Iljinskaja under degraded field condition[J]. Forest Ecology and Management, 2021, 482: 118897.

Abstract

Cyclocarya paliurus (Batal.) Iljinsk is an important medicinal plant for treating chronic diseases, but it is difficult to obtain high yields when grown on low-fertility soil. Inoculation with soil beneficial microorganism has suggested an effective means of stimulating plant growth and secondary metabolite production, but effect on plant performance when competing degraded field condition remains unclear. We combined controlled laboratory experiments with field trials to investigate the effects of co-inoculation with phyto-stimulatory strains (*Azospirillum brasilense* and *Pseudomonas fluorescens*) and nutrient-enhancing strains (*Bacillus megaterium* and *Azotobacter chroococcum*). Bacteria were applied with organic fertilizer at different fertilizer levels, and we tracked effects on soil nutrient availability as well as *C. paliurus* morphological traits, photosynthesis, growth and bioactive compounds during cultivation on barren land. Amendment of beneficial microbes with organic fertilizer enhanced the soil nutrient availability with high fertilizer showing greatest stimulation under controlled conditions, with the medium fertilizer giving best results in improving plant performance in the field. All fertilization regimes expanded the 3D root architecture, and bacterial additions increased the proportion of lateral roots compared to a single organic fertilizer treatment, which led to higher nutrient uptake. Inoculations at medium fertilizing level modified the root system and increased the photosynthesis rate, nutrient acquisition and plant growth. The co-inoculation with *B. megaterium* and *P. fluorescens* at medium fertilizer level stimulated the accumulation of flavonoids and polysaccharides, while co-inoculation with *A. chroococcum* and *A. brasilense* at low fertilizing level facilitated the production of flavonoids and triterpenoids. The biosynthesis of secondary metabolites exhibited strong correlations with leaf C/N and C/P ratios. Thus, manipulation of bioactive compounds in *C. paliurus* leaves can be affected by internal nutrient balance, which is associated with reformed root system morphology that modulated by bacterial inoculation.

1 Introduction

Introducing beneficial microbial consortia shows promising potentials in maintaining soil fertility as well as improving plantation productivity under degraded land conditions. As effective soil-remediation agents, the beneficial microbes contribute to soil nutrient cycling, plant growth promotion, and resistance of soil-borne phytopathogens (Domenech et al. 2004, Xiong et al. 2017) by direct or indirect mechanisms, such as nitrogen fixation, phosphate solubilization, and antagonistic action against pathogens (Schlemper et al. 2018, da Silveira et al. 2019, Kousar et al. 2020). On the one hand, beneficial microbes such as plant growth-promoting bacteria (PGPB) can improve soil macronutrient availability by enhancing the activity of phosphatase, urease and nitrogenase (Wu

et al. 2012). On the other hand, the host root exudates drive the assembly of probiotics to participate in associative symbiosis with hosts (Bashan et al. 2004, Kuramae et al. 2020). These probiotics are able to induce the change of plant metabolisms via for instance alteration of internal nutrient balance (Xie et al. 2018a), photosynthetic rate and chlorophyll content (Mishra et al. 2020) or changes in root morphology (Wang et al. 2016).

PGPB play a key role in improving plant adaptabilities and inducing systemic phytochemical responses to mitigate environmental stresses (Etalo et al. 2018, Asghari et al. 2020). In this case, strains like *Azospirillum* sp. and *Pseudomonas* sp. are categorized as phyto-stimulatory strains and have been widely studied not only for their specialties in improving nutrient acquisition (*e.g.* nitrogen-fixing or phosphate-solubilizing activities), but also for their ability to contribute to synergistic metabolic activities with the host plant (Karthikeyan et al. 2009, Walker et al. 2012). These synergistic effects have been shown to affect metabolites across a range of different plant organs and host species, such as modulation of metabolites in maize root and stearidonic acid accumulation in *Buglossoides arvensis* seed by inoculating with *Azospirillum* sp. or *Pseudomonas* sp. (Walker et al. 2012, Novinscak and Filion 2019). With respect to nutrient-improving strains, *Bacillus* sp. and *Azotobacter* sp. are able to improve nutrient acquisition and help plants grow in poor soils (Saxena et al. 2013, Latef et al. 2020). Co-inoculation of different PGPB is a promising strategy that can provide host plants with multiple benefits (Karthikeyan et al. 2009), but the combined effects of phyto-stimulatory and nutrient-enhancing strains are still poorly understood.

Cyclocarya paliurus (Batal.) Iljinsk is an important woody medicinal plant belonging to the Juglandaceae family (Fang et al. 2011). Its leaves are often used in herbal teas or as a key ingredient in Chinese traditional medicines used to treat diabetes and hyperlipidemia (Zhai et al. 2018). The bioactive extracts from *C. paliurus* leaves are mainly comprised of flavonoids, triterpenoids, and polysaccharides, which contribute to protecting humans against chronic diseases by antidiabetic, antioxidant, and antimicrobic effects (Zhang et al. 2010, Wu et al. 2017). However, many *C. paliurus* plantations exhibit limited production of metabolites in the leaves under field conditions. Consequently, there is an increasing demand on developing forest management strategies to improve the metabolite yields in *C. paliurus* plantation to increase the medicinal value.

The application of PGPB as bio-fertilizers is gaining considerable attention, and such strategies have been applied to a range of forest and agricultural systems including medicinal plants such *Glycyrrhiza uralensis* (Xie et al. 2018b), *Juglans regia* (Yu et al. 2012), and *T. foenumgraecum* (Dadrasan et al. 2015). However, the efficiency of PGPB application highly depends on the efficient delivery of the target organisms and their survival in the soil (Rashid et al. 2016), which are affected by numerous abiotic and biotic factors, such as soil nutrients (Treseder 2008), organic matter (Tejada et al. 2008), and competition with native soil microorganisms (Backer et al. 2018). To optimize

growth promotion effects and biological productivity in forest ecosystems, the densities of applied inocula should also be investigated, and that optimal densities differ for different PGPB. For instance, 10^6-10^7 cells per plant of *Azospirillum brasilense* were required to obtain a positive effect on the plant, while 10^5-10^6 cells per gram of root are suitable for *Pseudomonas* sp. application (Bashan 1986, Haas and Defago 2005).

Our previous study monitored the effects of single and mixed PGPB inoculant on soil properties and their survival dynamics in the soil (Wang et al. 2019b), thus provided a reference for selecting appropriate PGPB combinations and inoculation period for this study. We also confirmed that the yield of bioactive compounds in *C. paliurus* leaves can be affected by the changes of internal nutrient stoichiometry (Wang et al. 2019c). However, the intermediate relationships between microbial inoculation and the change of metabolic profiles in *C. paliurus* leaves remain unknown, the responses of soil nutrient dynamics to different inocula populations and combinations are not clear. We hypothesized that the effects of inoculations on soil nutrient levels and plant growth would increase with increasing levels of fertilization, and the plant root could play an important role as the intermediate agent linking inoculation and the host plant performance. In this study, we aimed to (i) examine if these PGPB strains amended with different amounts of organic fertilizer can act synergistically to enhance soil nutrient availability by a soil incubation experiment to avoid the uncontrollable factors in the field; and (ii) evaluate the effects of mixed PGPB inoculation on *C. paliurus* growth, 3D root architecture, leaf nutrient stoichiometry, and the yield of metabolites by conducting a natural field experiment under different fertilizing levels, thus providing a perspective of understanding the relationship between plant metabolite, nutrients and morphological traits in response to microbial inoculations.

2 Material and methods

2.1 PGPB strains growth and organic fertilizer

The four PGPB strains used in the study, included two nutrient-enhancing strains (C: *Azotobacter chroococcum* HKN-5, M: *Bacillus megaterium* W17) and two phyto-stimulatory strains (B: *Azospirillum brasilense* CW903, F: *Pseudomonas fluorescens* W12), and these were used as couples in the following combinations (M and F; C and B). These strains have been reported with the ability to improve soil nutrient availability and plant growth, and none of these bacterial strains shows antagonistic effects against one another (Wang et al. 2019b). Each strain was grown in lysogeny broth medium (pH 7.0, 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) at 28 °C, shaking at 180 rpm for 24–26 h until an optical density (OD) of 0.9 at 600 nm, which corresponded to the log phase. The bacterial

population was examined in a lab using the plate count serial dilution method while experimenting on building a standard curve between optical density and bacterial quantities. The suspensions were adjusted to a final concentration of 1×10^8 colony forming units (CFU)·mL^{-1} for each strain based on OD_{600nm}. The organic fertilizer used in the controlled and field experiments is mainly comprised of chicken manure, straw, tea dross and mushroom dross, and its chemical characteristics are provided in Table S1.

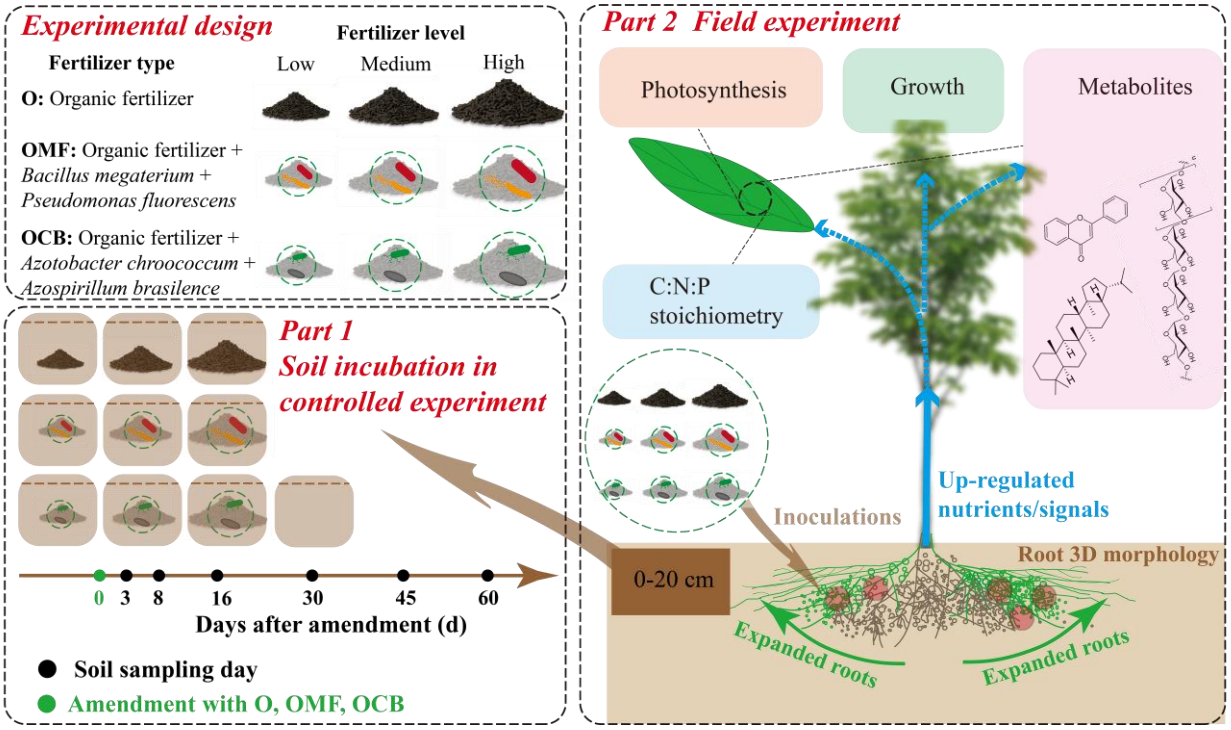


Figure 5.1 Conceptual map of experimental design, controlled experiment, and field experiment

2.2 Soil incubation in controlled experiment

The objective of the controlled experiment is to verify if the mixes of PGPB strains can synergistically enhance the soil nutrient properties with different level of organic fertilizer, and to test if the effect is persistent, which is hard to detect under field conditions. Soils for the incubation experiment were collected from five plots (depth: 0–20 cm, area: 1 × 1 m) in an "S" pattern at the *C. paliurus* plantation field. The soil is the representative type in subtropical regions in China, which was classified as clay loam soil with heavy texture and serious shortage of organic matter, pH 5.9, bulk density of 1.5 g·cm^{-3}, total C of 4.1 g·kg^{-1}, total N of 0.79 g·kg^{-1}, total P of 0.30 g·kg^{-1}, NH_4^+–N of 10.94 mg·kg^{-1}, NO_3^-–N of 2.68 mg·kg^{-1}, and available P of 1.03 mg·kg^{-1}.

After removing the plant material, stones and other debris, all soil samples were mixed thoroughly to form a composite sample and stored at 4 °C prior to use. Before incubation, the soils were amended with one of four organic fertilizer levels (none: 0 g·pot^{-1}, low: 14 g·pot^{-1}, medium: 28 g·pot^{-1}, high: 42 g·pot^{-1}), which equals to 0,

0.5, 1.0, 1.5 kg per plant in the field experiment, respectively (Table S2). After that, the soil mixtures were transferred to plastic pot (diameter × height: 8.5 × 8.4 cm, breathable and waterproof) with 350 gram in total per pot and sterilized to eliminate naturally occurring microbes.

After the former preparations, the soil mixtures were divided into two parts. One part was amended with two different PGPB combinations (OMF: *B. megaterium* and *P. fluorescens*; OCB: *A. chroococcum* and *A. brasilense*), and we set four different inoculating levels (none fertilizing control, low: 10^5 cell·pot^{-1}, medium: 10^6 cell·pot^{-1}, high: 10^7 cell·pot^{-1}) as in combination with four organic fertilizer levels, respectively. The other part of the soil mixture was regarded as single organic fertilizer treatment without PGPB addition (O: only organic fertilizer). Detailed information about experimental design is provided in Figure 5.1 and Table S2. Four replicates were set for each fertilizer type at each fertilizing level, giving a total of 40 pots for incubation. All the microcosms were incubated at 28 °C under dark condition for 60 days (Figure 5.1). During incubation, the soil moisture was held at 60% of the water holding capacity with sterile water.

2.3 Application of different fertilization regimes in field experiment

The objective of the field experiment is to seek the underlying relationships between different fertilization regimes, root morphology, growth performance and metabolite production of *C. paliurus*. The field study site is located in Baima (31°35′ N, 119°10′ E), Nanjing, China, which is a typical zone in the subtropics, with abundant rainfall (1037 mm/year) and sunshine (2146 h/year), and the annual average temperature being approximately 15.4 °C. The 2-year-old *C. paliurus* were set at a planting density of 2 × 2 m in the field (120 × 40 m) in 2015, with the same soil as described in the controlled experiment.

The field experiment was laid out in a three-block pattern based on completely randomized factorial design, with three fertilizer types (OMF, OCB, O) and four fertilization levels (none, low, medium, high; specifically: inoculants: 10^7, 10^8, 10^9 cells per plant; organic fertilizer: 0.5, 1.0, 1.5 kg per plant), giving a total of nine treatments and one control (non-fertilizing) in the field (Figure 5.1). The concentrations of inocula were approximately estimated according the results from the controlled experiment. Detailed information is provided in Table S2. Each treatment contained at least 60 healthy *C. paliurus* seedlings that were equally divided into three blocks. All inoculations were conducted on May 19th and July 13th, 2016 respectively. Briefly, we dug a 20-cm-depth circle around the plant vertical canopy projection, to get closer to the lateral root. Same procedures were also applied for the control without fertilization. The interval between two inoculations was set at 45 days according to the bacterial growth curve. The organic fertilizers were only implemented at the first inoculation time.

2.4 Soil sampling and analysis

In the controlled experiment, soils were vertically sampled on the 3rd, 8th, 16th, 30th, 45th, and 60th days of incubation using the hole-sampling method described before (Wang et al. 2019b). Briefly, three random vertical holes (diameter: 8 mm; depth: 60 mm) were implemented by sampling tube for each pot to lessen the disturbance of sampling on microbes, this resulted about 25-gram soil for each duplicate of each treatment. In the field, five soil samples (about 50 grams per sample) were collected randomly for each treatment in every block in September 2016. All samples were stored at 4 °C prior to following analyses.

Soil available N (SAN) was determined by extraction with 2M KCl in 1:5 (w/v) soil-to-solution ratio, shaking for 60 min at 200 rpm, and followed by quantification using a continuous flow analyzer (Bran + Luebbe AA3, Germany). Soil available P (SAP) was extracted by ammonium fluoride and hydrochloric acid in 1:10 (w/v) and determined using the molybdenum-blue method (Olsen 1954). Soil acid phosphatases activity (Acpase) was assessed using the method described by Tabatabai and Bremner (1969). Soil nitrogenase activity was measured by the acetylene reduction method (David et al. 1980).

2.5 Plant growth and 3D root architecture

Plant growth was evaluated as seedling height and stem basal diameter, which were measured for all healthy seedlings (20 plants for each treatment in each block) in March and September, 2016, respectively. The net growth of plant height and basal diameter were also calculated as the difference between the initial and the last value. For the measurement of leaf biomass, the fresh leaves of three plants were harvested entirely for each treatment in each block. Afterwards, the samples were dried at 60 °C and weighed for dry biomass.

To investigate the difference between seedlings under different fertilizer types, we selected three seedlings of each fertilizer type at medium fertilizing level (according to preliminary results) to compare 3D root morphological traits. Briefly, the whole roots of three seedlings for each treatment were carefully dug out and washed gently in September, 2016. A Trimble TX8 3D Scanner (©2012-2013, Trimble Navigation Limited, Version 1.00, USA) was used to scan the roots at three different positions. After that, we collected the data and analyzed the point clouds of roots in CloudCompare (v2.10-alpha, www.cloudcompare.org), which was able to calculate the C2C (cloud-cloud) distance and intensity after merging these roots in the same position (Girardeau-Montaut 2016). With such method, we were able to compare the differences of root clouds between different treatments based on cloud-cloud distance and cloud intensity.

2.6 Gas exchange parameters

Measurements for stomatal conductance, photosynthetic rate, transpiration rate, and intercellular CO_2 concentrations were performed using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE, USA) equipped with red and blue LED light sources on five plants per treatment at the same day. The measurements were performed between 09:00–10:00 a.m.

2.7 Leaf sampling and C: N: P stoichiometry

The fresh fully developed leaves were harvested at the same direction in September 25, 2016, with three plants for each treatment in each block. After that, all samples were dried at 60 °C, ground into powder and stored at room temperature for subsequent measurement of nutrients and bioactive compounds.

For the measurement of total carbon (C) and nitrogen (N) contents, each sample (50.0 mg) of leaves was wrapped up in aluminum foil and total C and N were determined by the elemental analyzer (vario MAX CN, Elementar, Hanau, Germany). For the measurement of total phosphorus (P) content, each sample (1 g) was digested by HNO_3 and $HClO_4$ (5:1 in volume), and total P was determined by the molybdenum-blue method. C/N ratio, C/P ratio and N/P ratio were then calculated.

2.8 Determination of bioactive compounds

For the measurement of bioactive compounds in the leaves, 9 samples for each treatment were used. Flavonoids were extracted from *C. paliurus* leaves using an ultrasonic-assisted method with 75% ethanol after removing fat-soluble impurities with petroleum ether. The total flavonoid concentration was determined using a colorimetric method with detection at 415 nm (Bao et al. 2005) and was calculated using the standard Rutin curve and expressed as a milligrams Rutin equivalent per gram of dry mass (mg/g).

The extraction of water-soluble polysaccharide in *C. paliurus* leaves was carried out as described previously by Fu et al. (2015), and the polysaccharide concentration was determined by the phenol–sulfuric acid method. For triterpenoid extraction, 2.0 g of leaves were extracted using an ultrasonic-assisted method. Briefly, 50 mL of 75% ethanol was added to each sample, and the extraction was conducted for 45 min at 65 °C and repeated twice. The total triterpenoid concentration was determined according to a previously described laboratory procedure using a colorimetric method (Fan and He 2006). The yields of these bioactive components in leaves were calculated as the concentration multiplied by the biomass of leaves.

2.9 Statistical analysis

The Shapiro-Wilk test and Levene's test were used to test the normal distribution of data and homogeneity of variances, respectively. Mixed linear model analysis was used to assess the effects of the fertilizer type, fertilizer level, soil incubation time, and their interactions on the soil's biochemical properties in the controlled experiment. Two-way ANOVA platform developed by Assaad et al. (2015) was used to estimate the effects of fertilizer type, fertilizer level and their interactions on soil properties at each sampling time point. Two-way ANOVA was also used to test the effects on plant growth, nutrient acquisition and the accumulation of bioactive compounds in the field experiment. Duncan's multiple range test was applied to determine the differences among individual treatment means. Tamhane's T_2 was used to test for differences among treatments when variances of tested data were not equal. Polynomial regression analysis was used for understanding the relationship between soil acid phosphatase activity and soil C/N ratio, and the relationship between the accumulation of bioactive compounds and leaf stoichiometric traits. R-square values were used for evaluate the fitness of the regression curve, and the F-test was applied to test if polynomial model provided a significantly better fit than the intercept-only model. All statistical analyses were considered significant at $p < 0.05$ and were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). The figures were plotted by GraphPad Prism (v.8.0.0, GraphPad Software, San Diego, California USA).

3 Results

3.1 Soil incubation experiment

Based on the linear mixed model analysis, the effects of the fertilizer type, fertilizer level, incubation time, and their interactions on soil biochemical properties are presented in Table S3. Clearly, all factors showed significant impacts on soil variables and exhibited significant interactions. We further compared the influences of fertilizer factors on soil nutrient contents at each sampling time point (Table S4). Fertilizer level showed significant impacts on soil available P and N at each time point, while fertilizer type presented no significant impacts on soil available P until 30d. As presented in Figures 2 and 3, soil nutrient contents significantly increased with the elevated fertilizing level. With the extension of culture time, the effects of bacterial addition on soil nutrients were stable and significant. Furthermore, soil inoculated with beneficial microbes (treatment OMF and OCB) exhibited higher nutrient contents than non-inoculated soils (treatment O). In terms of different soil available nutrient types, significant differences ($p<0.05$) between treatments OMF and OCB were observed in the last 15 days. Inoculation

with *B. megaterium* and *P. fluorescens* at medium level better improved soil available P content, while inoculation with *A. chroococcum* and *A. brasilense* at high level resulted in increased available N content in soil.

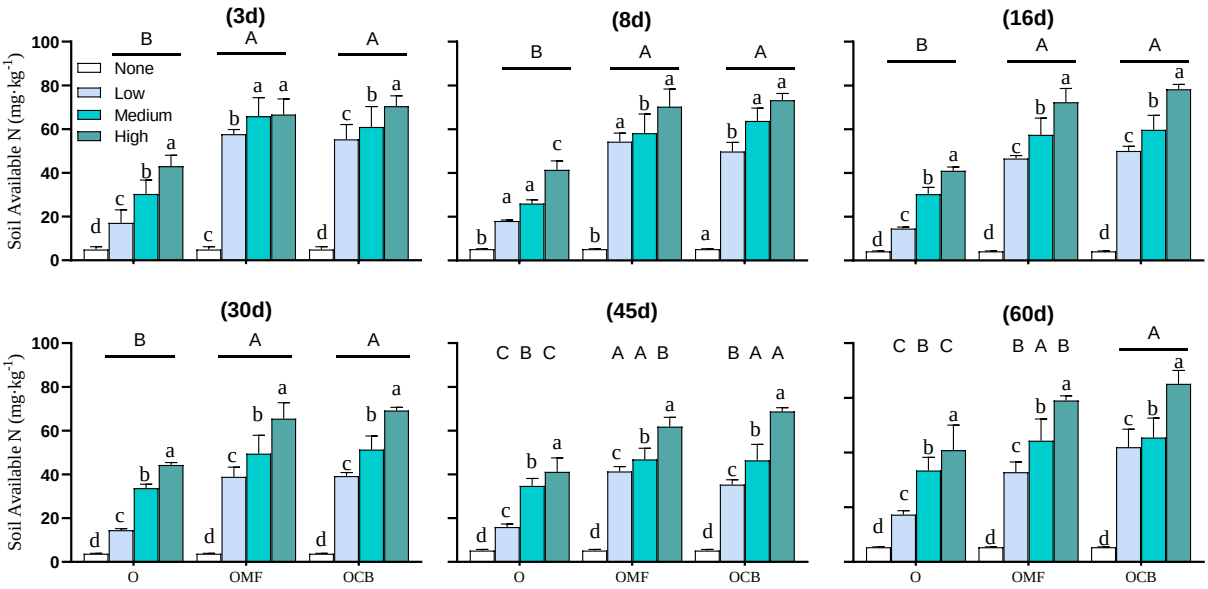


Figure 5.2 Effects of different fertilizing regimes on the available soil nitrogen contents at 3, 8, 16, 30, 45, and 60 days after first fertilization. The treatments are O: only organic fertilizer; OMF: organic fertilizer and inoculants containing both *Bacillus megaterium* and *Pseudomonas fluorescens*; OCB: organic fertilizer and inoculants containing both *Azotobacter chroococcum* and *Azospirillum brasilense.* The lowercase letters indicate significant differences between fertilizing levels ($p<0.05$) within the same fertilizer type; the capital letters indicate significant differences between fertilizer types ($p<0.05$) within the same fertilizer level.

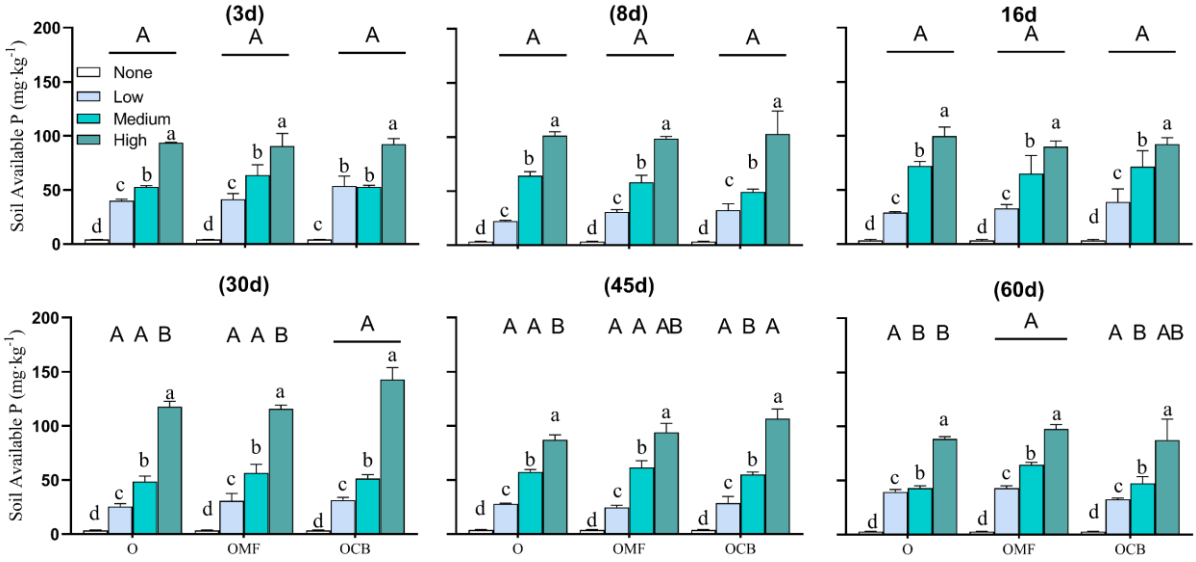


Figure 5.3 Effects of different fertilizing regimes on the available soil phosphorus contents 3, 8, 16, 30, 45, and 60 days after first fertilization. The treatments are O: only organic fertilizer; OMF: organic fertilizer and inoculants

containing both *Bacillus megaterium* and *Pseudomonas fluorescens*; OCB: organic fertilizer and inoculants containing both *Azotobacter chroococcum* and *Azospirillum brasilense.* The lowercase letters indicate significant differences between fertilizing levels ($p<0.05$) within the same fertilizer type; the capital letters indicate significant differences between fertilizer types ($p<0.05$) within the same fertilizer level.

Soil enzyme activities showed similar trends as observed for nutrients, but were affected by different factors (Table S5, S6). Soil acid phosphatase activities showed a strong dependency on fertilization level, with higher fertilization levels resulting in higher acid phosphate activities (Table S5). On the other hand, nitrogenase activities were affected by incubation time: values increased from 3rd day to 30th day, but decreased thereafter (Table S6, Figure 5.4d). To further examine the relationship between nutrients and enzyme activities under different conditions, we analyzed the correlations between them under different fertilizing levels, sampling time points, and C/N ratios (Figure 5.4). It appeared that available soil nutrients were not only positively correlated with enzyme activities ($p<0.01$), but also affected by fertilizing level. Overall, soil acid phosphate activities related to the change of soil C/N ratio (Figure 5.4c), but soil nitrogenase activities exhibited a preference for the change of incubation time (Figure 5.4d).

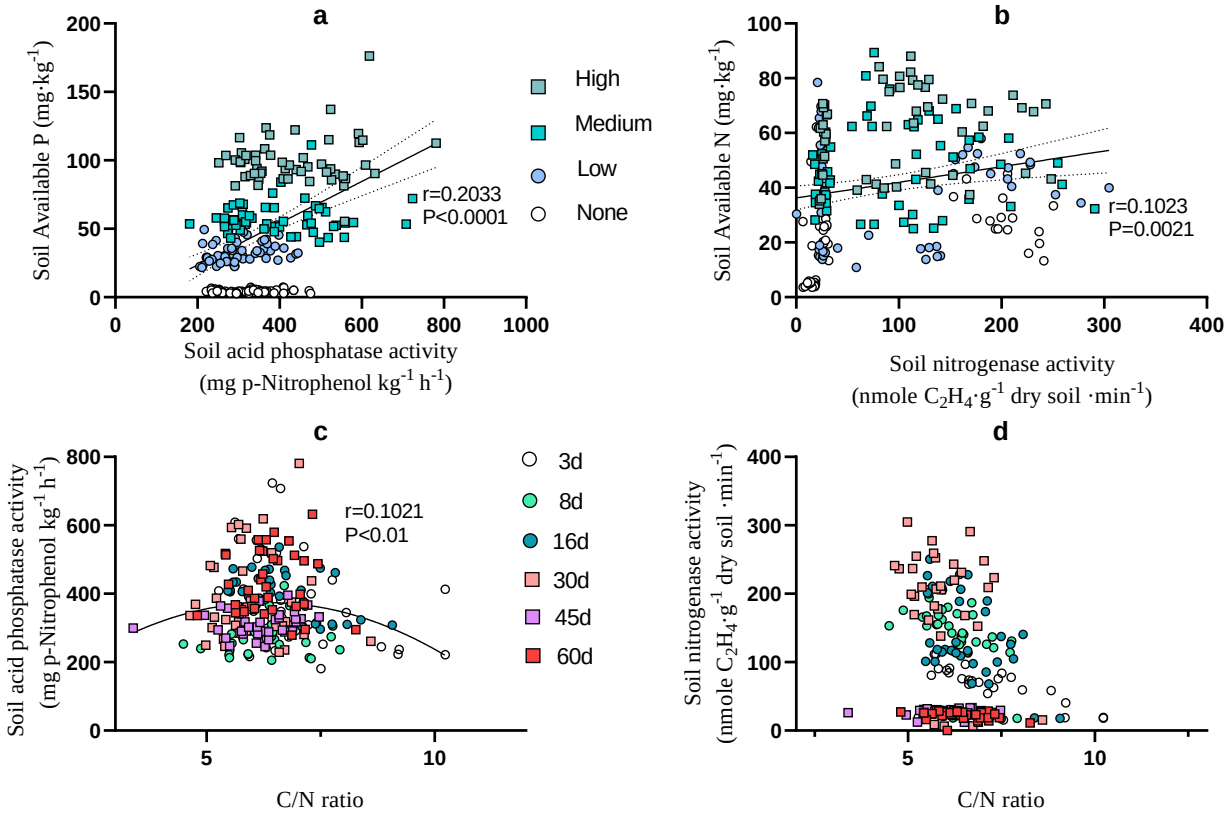


Figure 5.4 The relationships between (**a**) acid phosphatase activities (Acpase) and soil available phosphorus (SAP) contents under different fertilizing level, (**b**) nitrogenase activities and soil available nitrogen (SAN) contents under different fertilizing level, (**c**) Acpase and soil C/N ratio in different sampling time points, (**d**) nitrogenase and soil C/N ratio in different sampling time points.

3.2 The effects on the plant performances in field experiment

3.2.1 Net growth of plant height and basal diameter

Bio-fertilization significantly increased the net plant growth (the difference between initial and final measurements) during the investigation, both in terms of height, basal diameter, and leaf biomass, in comparison with the control (Figure 5.5, $p<0.05$). Both fertilizer type and fertilizer level played significant roles in regulating plant growth (Table S7). Hence, different combinations of fertilizer types and levels resulted in different outcomes of *C. paliurus* growth. For instance, all fertilizer types at the medium level performed better than other fertilizing levels in improving net growth parameters ($p<0.05$). When comparing different fertilizer types at the same fertilizing level, the inoculation of beneficial microbes accompanied by organic fertilizer (treatment OMF and OCB) resulted in better growth than single organic fertilizer treatment (O).

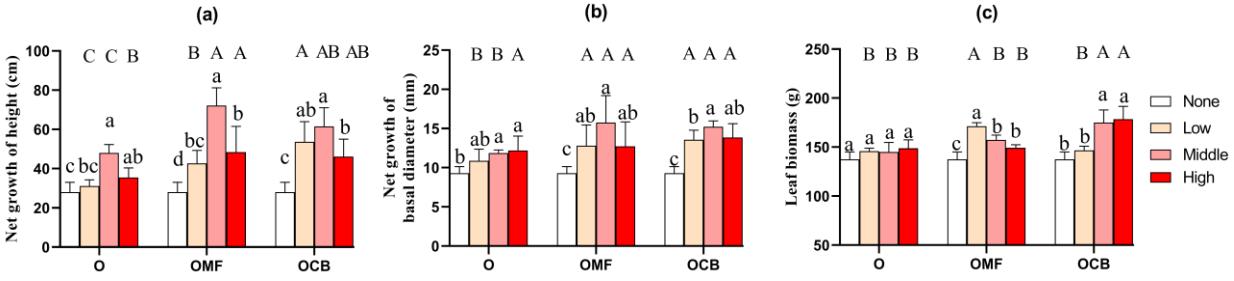


Figure 5.5 Net plant growth as measured by the differences of plant height (**a**) and stem basal diameter (**b**) between initial and final measurements. The treatments are O: only organic fertilizer; OMF: organic fertilizer and inoculants containing both *Bacillus megaterium* and *Pseudomonas fluorescens*; OCB: organic fertilizer and inoculants containing both *Azotobacter chroococcum* and *Azospirillum brasilense.* The lowercase letters indicate significant differences between fertilizing levels ($p<0.05$) within the same fertilizer type; the capital letters indicate significant differences between fertilizer types ($p<0.05$) within the same fertilizer level.

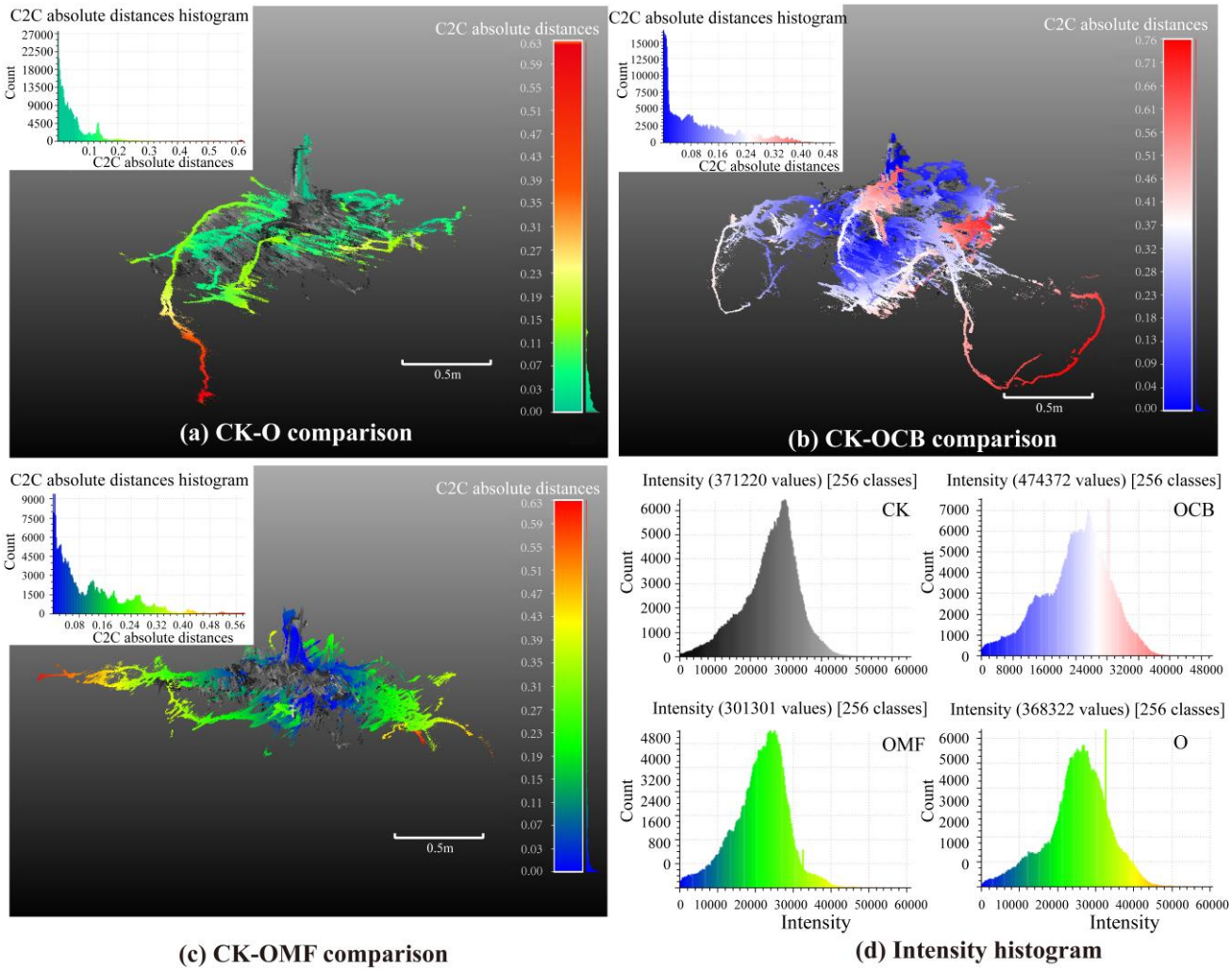


Figure 5.6 3D root architecture of *Cyclocarya paliurus* under different fertilization regimes and their absolute C2C (cloud to cloud) distances. The histogram shows the values of points in different C2C distances. (**a**) The C2C absolute distance between treatment O (organic fertilization) and control (black root architecture); (**b**) The C2C absolute distance between treatment OCB (inoculation of *A. chroococcum* and *A. brasilense* accompanied by organic fertilizer) and control; (**c**) The C2C absolute distance between treatment OMF (inoculation of *B. megaterium* and *P. fluorescens* accompanied by organic fertilizer) and control; (**d**) the intensity of point cloud in different treatment.

3.2.2 3D Root architecture

To compare the impact of different fertilization regimes on root architecture, we calculated the absolute C2C (cloud to cloud) distances between point cloud of fertilized (at medium level) and non-fertilized seedlings (Figure 5.6). The 3D root architecture of all fertilized seedlings exhibited a wider and bigger cloud as compared to the control. However, the seedling roots in three fertilizer types showed different cloud shapes as well as C2C distances. The

horizontal distribution range of roots in OMF was the highest of all treatments (Figure 5.6c), while the cloud shape of OCB was much bigger than other treatments (Figure 5.6b). Furthermore, in comparison with single organic fertilization, the inoculation of different strains increased the number of cloud points that were in a higher range of C2C distance (Figure 5.6a, b, c). It must be mentioned that the inoculation not only impacted root distribution, but also changed the proportion of lateral roots versus thick roots, which could be reflected in the changes of intensity (Figure 5.6d). Compared to the cloud intensity of roots under the no fertilizer control, treatment OCB resulted in higher counts of points in the intensity range from 20000 to 40000. Also, the integral area from the intensity of 0 to 20000 was increased in treatments OCB and OMF, indicating a higher predominance of lateral small roots in these inoculated seedlings.

Table 5.1 Gas exchange parameters for *Cyclocarya paliurus* leaves under different fertilization regimes.

Fertilizer type	**Fertilizer level**	**Transpiration rate (mmol m^{-2} s^{-1})**	**Stomatal conductance (mmol m^{-2} s^{-1})**	**Photosynthetic rate (μmol s^{-1} m^{-2} leaf area)**	**Intercellular CO_2 concentration (μmol mol^{-1})**
O	None	2.34±0.18d	39.40±4.94e	3.65±0.24e	344.00±14.48a
	Low	2.74±0.13c	52.60±3.13d	4.25±0.10d	295.10±17.63b
	Medium	2.94±0.22abc	88.00±3.12b	4.60±0.46cd	342.50±19.65a
	High	2.88±0.14bc	63.60±5.42c	4.45±0.37cd	289.60±11.24b
OMF	None	2.34±0.18d	39.40±4.94e	3.65±0.24e	344.00±14.48a
	Low	3.22±0.24ab	96.00±7.25ab	6.05±0.21b	258.80±10.07cd
	Medium	3.36±0.19a	96.33±5.36ab	7.25±0.88a	254.10±1.88d
	High	3.06±0.07b	82.40±7.39b	4.97±0.61bc	281.30±10.35bc
OCB	None	2.34±0.18d	39.40±4.94e	3.65±0.24e	344.00±14.48a
	Low	3.14±0.14ab	94.00±6.58ab	5.92±0.34b	261.80±4.32c
	Medium	3.30±0.09a	107.50±8.14a	6.40±0.47ab	278.20±6.09bc
	High	3.29±0.12a	103.00±10.21a	6.85±0.42a	255.40±2.17d

3.2.3 Gas exchange parameters

Both fertilizer type and fertilizer level significantly affected plant photosynthesis rates (Table 5.1, Table S7). In

comparison with organic fertilization (O), inoculation with beneficial microbes at certain fertilizer levels performed better in enhancing photosynthesis rate. The photosynthesis rate presented the highest value in treatment OMF at a medium fertilizer level (98% higher than the control), followed by the OCB treatment at medium and high fertilization levels (75%, and 87% higher than the control, respectively). Transpiration rate and stomatal conductance in leaves were also promoted by inoculation, while intercellular CO_2 concentrations in leaves was lower than observed for the control.

Table 5.2 The total contents of carbon, nitrogen, phosphorus and their ratios in *C. paliurus* leaves under different fertilization regimes.

Fertilizer type	Fertilizer level	Carbon ($g \cdot kg^{-1}$)	Nitrogen ($g \cdot kg^{-1}$)	Phosphorus ($g \cdot kg^{-1}$)	C/N	C/P	N/P
O	None	459.5±38.6	20.15±0.29e	1.59±0.05d	22.82±1.5a	288.96±2.2a	12.62±0.7b
	Low	466.2±40.5	22.35±0.65cd	1.75±0.05bc	20.84±2.2abc	266.45±2.9bc	12.72±0.6b
	Medium	463.3±11.13	22.86±0.59cd	1.68±0.06cd	20.22±0.8c	275.75±3.4ab	13.66±0.8ab
	High	466.6±27.4	23.11±0.43c	1.69±0.04c	20.18±0.47c	276.23±2.5ab	13.62±1.1ab
OMF	None	459.5±38.6	20.15±0.29e	1.59±0.05d	22.82±1.5a	288.96±2.2a	12.62±0.7b
	Low	459.7±62.7	23.02±0.36c	1.88±0.11ab	19.92±1.0cd	244.51±5.9de	12.28±1.2bc
	Medium	465.1±15.1	23.01±0.46c	1.86±0.07ab	20.21±0.2c	250.05±6.2de	12.37±1.2bc
	High	458.4±48.6	25.10±0.21b	1.84±0.05b	18.24±0.7d	249.16±7.5de	13.67±0.9ab
OCB	None	459.5± 38.6	20.15±0.29e	1.59±0.05d	22.82±1.5a	288.96±2.2a	12.62±0.7b
	Low	460.8±16.0	21.37±0.44d	1.76±0.05bc	21.56±0.8ab	261.80±3.4cd	12.14±0.1bc
	Medium	459.8±15.8	27.74±0.61a	1.98±0.07a	16.55±0.2e	232.83±2.7e	14.05±0.6a
	High	462.2±25.1	25.62±0.76ab	1.81±0.06b	18.09±0.4d	255.36±9.9cd	14.12±0.4a

3.2.4 C: N: P stoichiometry in C. paliurus leaves

All fertilization regimes significantly affected the contents of nitrogen and phosphorus in *C. paliurus* leaves ($p<0.05$, Table 5.2, S7) compared to the control. Compared to the single application of organic fertilizer, inoculation with beneficial strains further improved plant nutrient contents. However, the stoichiometry of nutrient accumulation differed between treatments. For instance, inoculation with OCB at medium and high fertilizer levels yielded the

highest nitrogen content, which in turn resulted in the lowest C/N ratio. Inoculation with OCB at the medium fertilizer level and OMF at low and medium levels significantly increased phosphorus contents in the leaves.

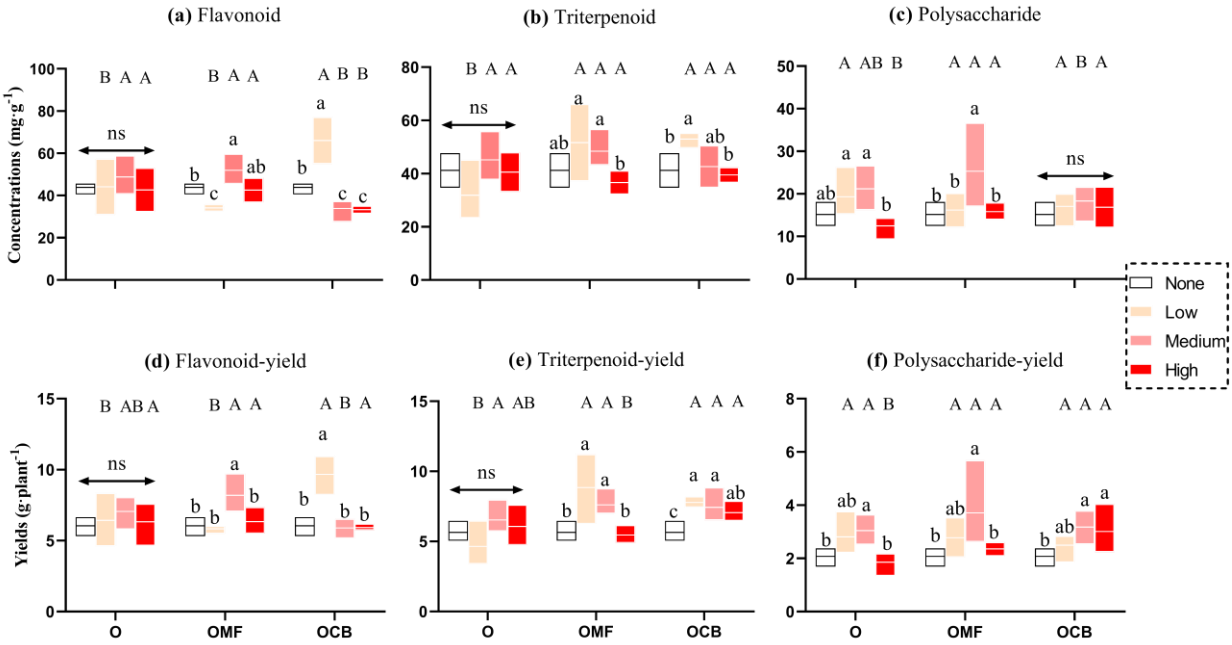


Figure 5.7 Concentration and yield of flavonoids, triterpenoids, and polysaccharides under different fertilization regimes.

3.2.5 Bioactive compounds

The concentration and yield of bioactive compounds (BC) under different fertilization regimes are shown in Figure 5.7. It appeared there was a strong dependence of BC concentrations and yields on fertilizer type and level. For instance, in comparison to the control, combined inoculation with organic fertilizer and beneficial strains (treatment OMF and OCB) significantly altered the BC accumulation in *C. paliurus* leaves, while no changes of flavonoid and triterpenoid were observed in the single application of organic fertilizer (treatment O).

When comparing different fertilizer types and levels, the highest concentrations of flavonoid and triterpenoid were both observed in treatment OCB at the low fertilization level (Figure 5.7a, b). Also, treatment OMF at the medium fertilization level increased the concentration of flavonoids and polysaccharides, which were 18% and 67% higher than that in control ($p<0.05$), respectively. However, it must be noted that the other inoculations decreased the concentration of flavonoids in comparison to the control ($p<0.05$), and no significant change was found in the concentrations of triterpenoids and polysaccharides. Most bio-fertilization regimes increased the yield of BC compared to the control. Among these treatments, OMF at medium fertilizer level and OCB at the low level of

fertilization resulted in higher flavonoid and triterpenoid yields (Figure 5.7d, 7e), while the yields of polysaccharides were significantly increased by all fertilizer types at medium level ($p<0.05$, Figure 5.7f).

3.2.6 The relationship between internal nutrient balance and bioactive compound accumulation

To examine the relationship between internal nutrient balance and BC accumulation in *C. paliurus* leaves, we used scatter plots to fit the C/N ratio, C/P ratio with yields and concentrations of BC. The concentrations and yields of flavonoids under different C/N and C/P ratios are shown in Figure 5.8. A clear pattern was emerged in which the highest accumulation of flavonoids was observed at median C/N and C/P values, regardless of the concentrations and yields. The F-test result showed that polynomial model provided a significantly better fit than the intercept-only model ($p<0.05$). According to the R-squared of each fit curve, the C/N ratio (Figure 5.8a) may play a more important role than the C/P ratio (Figure 5.8b) in regulating the accumulation of flavonoids. However, no clear relationship was detectable between triterpenoids, polysaccharides, and nutrient balance (data not shown).

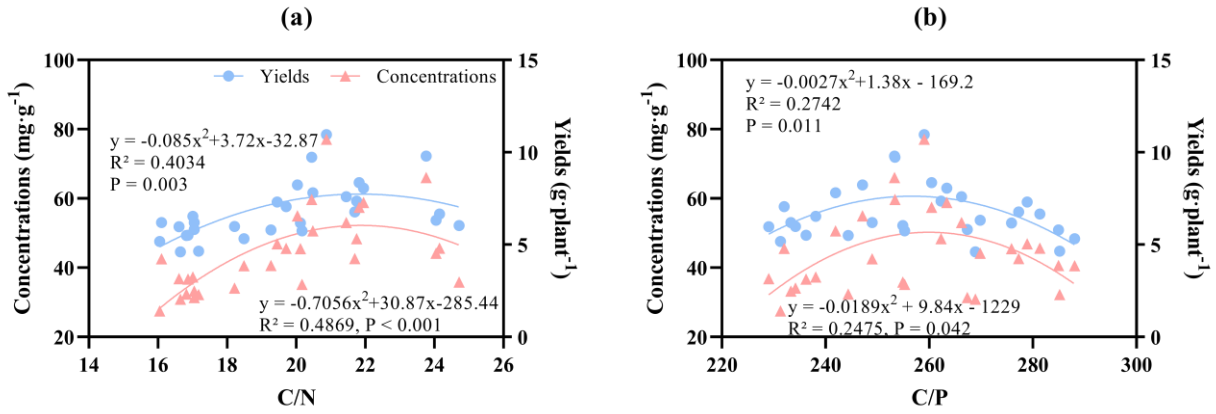


Figure 5.8 Scatter plots with fit curve between the C/N ratio, C/P ratio and the yields and concentrations of flavonoids in *C. paliurus* leaves.

4 Discussion

Beneficial microorganisms have been touted as an important tool to increase the efficiency of organic fertilizer under low-fertility soil conditions. In the present study, we added beneficial bacterial inoculants in the soil collected from the field with and without organic fertilizer, in order to assure the effects of inoculation and to avoid uncontrolled factors in the field. Compared to non-inoculated soils (control and treatment with only organic fertilizer), co-inoculation with beneficial strains and organic fertilizer synergistically enhance the soil nutrient

availabilities and enzyme activities (Figures 2, 3), which is also in accordance with previous studies (Yu et al. 2019). Organic fertilizer serves as a source of available carbon source for bacterial proliferations (Picart et al. 2016), and the beneficial microbes may exert multiple effects on soil such as impacts on nitrogen fixation, iron sequestration and phosphorus solubilization (Rashid et al. 2016). Interestingly, the enzyme activity that related to the available nutrient was also influenced by fertilizing level and incubation time (Figure 5.4). Both fertilizing level and inoculation period are important factors that should be considered for practical application. Fertilizing levels determinate the contents of organic matter input and microbial populations in the soil, thus affects the enzyme activity. Incubation time impacted the survival of beneficial microbes and the microbial efficiency on the soil (Strigul and Kravchenko 2006).

Important morphological traits in the field experiment, like plant height and basal diameter, are affected by numerous biotic and abiotic factors. Based upon our controlled experiment, we hypothesized that the effects of inoculation on the plant growth in the field would increase with the elevated fertilization levels. However, we observed that all fertilizer types at the medium level performed better than other fertilizer levels in terms of improving net growth parameters (Figure 5.5). Similar results have been reported recently in which maximum plant height was observed with a medium dose of PGPB inoculants, while the highest level treatments slightly reduced plant growth (Kumar et al. 2020). A possible reason could be that the plant exhibits less reliance on the soil microbes when we offered more nutrients by increasing the fertilizing, thus decreasing the possibility of synergistic interactions between organic fertilizer and soil beneficial microbes (Altieri and Nicholls 2003). On the contrary, a specific level of organic fertilizer and inocula could act coordinately to improve soil environment as well as plant growth. Also, an optimal level of organic fertilizer input is helpful for the establishments of beneficial microbes for instance by influencing the amount of available carbon and the soil C/N ratio (Defez et al. 2019), as supported the results in the controlled experiment in which enzyme activities were affected by the C/N ratio in a specific range of 5.5 – 7.5.

We also compared the 3D root morphology of *C. paliurus* when grown with different fertilizer types at the medium level of fertilization (Figure 5.6). Compared to traditional method of investigating 2D root morphology, visualizing 3D root architecture can track the whole root development in soil where various biotic and abiotic interactions actually take place (Metzner et al. 2015). Inoculations, especially for nitrogen fixers *Azotobacter chroococcum* and *Azospirillum brasilense* mixed inoculants, expanded the root distribution (increased the lateral roots) and changed the intensity of the root point cloud. The genus *Azospirillum* has been shown to contain species that can stimulate root system growth and improve root morphological efficiency (Rondina et al. 2020) for instance via the production

of auxins, ethylene, and nitric oxide in the rhizosphere, and these growth regulators and signals contribute to the root morphological feedbacks (Molla et al. 2001, Fukami et al. 2018). The observed longer and branched root systems as induced by inoculations can help the plant to obtain a greater access to soil microsites and provide more efficient nutrient acquisition in low-fertility soils (Comas et al. 2012).

Investigating nutrient content and C: N: P stoichiometry in *C. paliurus* leaves provides plant internal chemical traits after different fertilization regimes. It appeared that the contents of nitrogen and phosphorus increased in all treatment, with a better performance found in seedlings under inoculations compared to organic fertilization. This is in accordance with the soil nutrient results observed in the controlled experiment, indicating the inoculants with organic fertilizer increased the soil nutrient availabilities and nutrient acquisitions in the host plant (Shahzad et al. 2014, Yu et al. 2019). The co-inoculation of beneficial microbes and organic fertilizer performed better in stimulating soil nutrients and plant growth as compared to the single organic fertilizer treatment, indicating the synergistic effects between organic matter and inoculants in helping facilitate nutrient cycling in the soil and growth promotion of the host plant (Song et al. 2015). As expected, co-inoculation with *A. chroococcum* and *A. brasilense* (treatment OCB) at a medium fertilizer level significantly increased the N and P contents (Table 5.2), which is in line with results found for root morphology (Figure 5.6). Thus, the reformed root system induced by co-inoculation with *A. chroococcum* and *A. brasilense* could contribute to the observed changes in internal nutrient status. It has been reported that inoculation with *A. brasilense* increased the percentage of the small roots (< 0.50 mm) (Rondina et al. 2020), suggesting a higher capacity for nutrient acquisition and a plastic turnover of the root system to adapt to soil managements (Zangaro et al. 2014, Moretti et al. 2020a).

To evaluate the performance of medicinal plants in response to bio-fertilizations, it is also important to consider the physiological responses of the aboveground part of the plant, especially with respect to gas exchange parameters and bioactive compounds in the leaves. We found that co-inoculation of phosphate solubilizing bacteria *B. megaterium* and *P. fluorescens* at the medium fertilizer level resulted in the highest photosynthesis rate, followed by co-inoculation of nitrogen fixers *A. chroococcum* and *A. brasilense* at the medium fertilizer level (Table 5.1). Photosynthesis is a fundamental physiological process impacting biomass accumulation and organ formation in green plant species (Galle and Feller 2007). Seedlings with higher photosynthesis obtained a higher net growth after inoculation. More importantly, the primary metabolisms affected by photosynthesis may in turn influence the biosynthesis of secondary metabolite production (Zhu et al. 2017). We found that co-inoculation of *B. megaterium* and *P. fluorescens* at the medium fertilizer level promoted photosynthesis as well as the accumulation of flavonoids and polysaccharides. However, it must be noted that gas exchange parameters reflect what is

occurring at a single moment, thus should be considered with other leaf parameters to reveal the relation to metabolites accumulation. Other factors besides photosynthesis, such as internal nutrient balance, are also important in determining the biosynthesis of secondary metabolite.

The flavonoids, triterpenoids, and polysaccharides in *C. paliurus* leaves are responsible for numerous medicinal effects, and the biosynthesis of these bioactive compounds is influenced by genetic, cultivation practices and climatic factors (Fu et al. 2015, Liu et al. 2018b). To optimize the medicinal value of *C. paliurus*, it is important to apply plantation management systems that serve to increase the yield of target bioactive compounds in the leaves (Salla et al. 2014). In the present study, beneficial microbial inoculants and organic fertilizer were utilized as soil remediating agents to improve the growth and yield of *C. paliurus*. Co-inoculation of *A. chroococcum* and *A. brasilense* at the low fertilizer level could enhance the concentration of flavonoids and triterpenoids, while co-inoculation of *B. megaterium* and *P. fluorescens* at the medium level increased the concentration of flavonoids and polysaccharides. The interplay between the host plant and soil beneficial microbes could facilitate the phytochemical accumulation to alleviate environmental stresses (low fertility in this study) (Zade et al. 2019). Previous study has revealed that the plant secondary metabolite production is also regulated by internal nutrients balance (Lillo et al. 2008). As discussed above, alterations in root system morphology after PGPB inoculation can lead to altered internal nutrient balance, which could play an important role in regulating plant metabolism. Carbon, nitrogen and phosphorus metabolisms are intimately tied in almost every biochemical pathway within plants, thus the changed internal nutrient stoichiometry (C/N, C/P) could influence both primary growth and secondary metabolite production (Gigolashvili and Kopriva 2014, Canovas et al. 2018). Our correlation analysis showed that the accumulation of flavonoids in *C. paliurus* leaves was linked to the C/N and C/P ratios. At the appropriate C/N (~ 20-22) and C/P ratios (~ 250-260), the optimal yield and concentration of flavonoids could be obtained. This is supported by the nutrient uptake results of the OCB treatment at the low fertilizer level and OMF treatment at the medium level (Table 5.2). This finding was also partially agreed with the predication of growth-differentiation balance (GDB) hypothesis (Stamp 2003, 2004), which indicate the plants should allocate more resource to secondary metabolisms when experiencing intermediate resource level. However, the other treatments with a similar C/N or C/P ratio did not increase flavonoid production. Hence, soil beneficial microorganisms may modulate the biosynthesis of secondary metabolites by a range of mechanisms factors after their inoculation, such as changes in gene expression (Lillo et al. 2008), enzyme activity (Deng et al. 2019b) or phytohormone levels (Salla et al. 2014, Ravanbakhsh et al. 2019a). It must be noted that the greater concentration of bioactive compounds under stress conditions may lead to a compromise with respect to biomass yield. Hence,

to achieve an optimal yield of target ingredients, the relationship between leaf production and phytochemical concentration should be balanced when the plantation is designed for medicinal production.

5 Conclusion

Our results revealed that manipulation of bioactive compounds in *C. paliurus* leaves was affected by internal nutrient balance, which was associated with changes in root system after microbial inoculation. Application of PGPB consortia could result different effects between controlled and field conditions: inoculation increased the soil nutrient availability with increasing fertilization level in the lab, but the medium fertilizer level provided optimal growth in the field. To enhance the effects on the host plant growth, co-inoculations at medium fertilization level can expand lateral root distribution, increase photosynthesis rates, and facilitate nutrient acquisition. To achieve an optimal yield of medicinal ingredients, co-inoculation with *B. megaterium* and *P. fluorescens* at a medium fertilizer level can stimulate the accumulation of flavonoids and polysaccharides, while co-inoculation with *A. chroococcum* and *A. brasilense* at low fertilizer level can facilitate the production of flavonoids and triterpenoids. Hence, both fertilizing type and level should be considered when meeting various cultivation objectives. To further investigate the relationship between these strains and bioactive compounds, co-inoculation with four strains may be a promising strategy for future studies. Overall, these results revealed that microbial inoculation may modulate the biosynthesis and production of secondary metabolites by altering the root morphology and the C/N, C/P in the leaves, thus provided a sustainable and eco-friendly strategy to optimize the yield while balancing the growth under degraded land.

Supplementary material

Table S1. The chemical properties of organic fertilizer used in this study.

Soil properties	pH	Total organic matter	Total N	C/N ratio	P_2O_5	K_2O
Contents	6.68	41.20%	1.70%	24.2	0.8%	1.1%

Table S2. Fertilization regimes in controlled experiment and field experiment.

Experiment			**Field**				**Controlled**		
Treatment			O[a]	OMF[b]	OCB[c]		O	OMF	OCB
Strain			/	M & F	C & B		/	M & F	C & B
Fertilizing levels	None		/	/	/		/	/	/
	Low	PGPR[d] (cell·plant^{-1})	/	10^7	10^7	PGPR (cell·pot^{-1})	/	10^5	10^5
		O (kg·plant^{-1})	0.5	0.5	0.5	O (g·pot^{-1})	14	14	14
	Medium	PGPR	/	10^8	10^8	PGPR	/	10^6	10^6
		O	1.0	1.0	1.0	O	28	28	28
	High	PGPR	/	10^9	10^9	PGPR	/	10^7	10^7
		O	1.5	1.5	1.5	O	32	32	32

[a] Organic fertilizer;

[b] Inoculation of *Bacillus megaterium* (M) and *Pseudomonas fluorescens* (F) accompanied by organic fertilizer;

[c] Inoculation of *Azotobacter chroococcum* (C) and *Azospirillum brasilence* (B) accompanied by organic fertilizer;

[d] Plant growth promoting rhizobacteria

Table S3 Summary of the linear mixed model for the effects of fertilizer type, fertilizer level, incubation time, and their interactions on soil characteristics in the controlled experiment.

Variables	Model parameters		Fertilizer Type		Fertilizer Level		Incubation time	
	R-square	P-value	F-test	P-value	F-test	P-value	F-test	P-value
SAP	0.953	0.00	1.83	0.06	1382.69	0.00	4.19	0.00
SAN	0.958	0.00	399.55	0.00	1184.59	0.00	28.49	0.00
Acpase	0.694	0.00	6.24	0.00	65.03	0.00	30.00	0.00
Nitrogenase	0.943	0.00	16.80	0.00	302.26	0.00	321.53	0.00
NH_4^+	0.968	0.00	1262.82	0.00	963.49	0.00	20.20	0.00
NO_3^-	0.918	0.00	15.71	0.00	589.06	0.00	70.91	0.00
C	0.946	0.00	7.43	0.00	1138.79	0.00	43.89	0.00
N	0.925	0.00	73.37	0.00	784.82	0.00	17.93	0.00
C/N	0.699	0.00	38.75	0.00	58.26	0.00	25.71	0.00
Variables	**Type × Level**		**Type × Time**		**Time × Level**		**Type × Level × Time**	
	F-test	P-value	F-test	P-value	F-test	P-value	F-test	P-value
SAP	1.94	0.04	1.84	0.06	11.36	0.00	1.73	0.02
SAN	46.71	0.00	8.71	0.00	4.42	0.00	1.72	0.02
Acpase	1.70	0.13	2.57	0.01	7.24	0.00	1.90	0.01
Nitrogenase	10.34	0.00	5.62	0.00	49.95	0.00	7.96	0.00
NH_4^+	142.41	0.00	4.28	0.00	1.91	0.03	1.41	0.10
NO_3^-	5.38	0.00	11.64	0.00	8.21	0.00	2.14	0.00
C	3.44	0.00	1.50	0.15	8.31	0.00	1.89	0.01
N	9.39	0.00	1.19	0.30	4.21	0.00	0.59	0.95
C/N	4.41	0.00	0.58	0.83	9.56	0.00	0.45	0.99

Table S4 The *P*-Values of factors fertilizer type, level, and their interaction effects on soil biochemical properties in the controlled experiment as analyzed by two-way ANOVA.

Sampling day	Variable	*P*-value			Variable	*P*-value		
		Fertilizer level	Fertilizer type	Type×Level[1]		Fertilizer level	Fertilizer type	Type×Level[1]
3	Soil available P	<0.001	0.373	0.029	Soil acid phosphatase	<0.001	0.09	0.032
8		<0.001	0.954	0.127		<0.001	0.061	0.745
16		<0.001	0.721	0.913		0.164	0.024	0.399
30		<0.001	0.051	0.03		<0.001	0.066	0.434
45		<0.001	0.047	0.005		0.003	0.001	0.011
60		<0.001	0.006	0.003		<0.001	0.2	0.165
3	Soil available N	<0.001	<0.001	<0.001	Soil nitrogenase	<0.001	0.291	0.004
8		<0.001	<0.001	<0.001		<0.001	0.103	0.396
16		<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
30		<0.001	<0.001	<0.001		<0.001	0.003	<0.001
45		<0.001	<0.001	<0.001		<0.001	0.006	0.004
60		<0.001	<0.001	0.004		<0.001	0.331	0.106

[1] Type × Level = Fertilizer Type × Fertilizer Level interaction effect.

Table S5 Soil acid phosphatase activities in all fertilization regimes under different sampling time points in the controlled experiment.

Sampling time (d)	Treatment	Acid phosphatase activity (mg p-Nitrophenol $kg^{-1}·h^{-1}$)			
		Fertilizing level			
		None	Low	Medium	High
3	O	285.86 b	259.30 cC	298.64 bC	434.77 aB
	OMF	285.86 c	295.38 cA	644.83 aA	415.71 bB
	OCB	285.86 c	279.16 cB	412.36 bB	527.66 aA
8	O	252.46 b	209.84 cB	290.54 aB	301.27 aB
	OMF	252.46 bc	268.54 bA	333.32 aA	330.24 aA
	OCB	252.46 c	219.86 dB	271.49 bC	309.37 aB
16	O	368.96 b	306.04 cC	385.38 aC	373.40 abB
	OMF	368.96 c	405.08 bB	467.62 aA	492.66 aA
	OCB	368.96 c	425.69 aA	424.58 aB	390.61 bB
30	O	301.43 b	236.82 cC	297.19 bC	531.32 aB
	OMF	301.43 c	319.97 cA	436.20 bB	596.08 aA
	OCB	301.43 c	267.14 cB	475.22 bA	554.26 aB
45	O	303.44 c	363.23 aA	325.47 bA	338.59 abA
	OMF	303.44 b	281.15 bcB	271.51 cC	329.42 aA
	OCB	303.44 b	276.47 dB	290.14 cB	340.62 aA
60	O	289.78 d	373.79 cA	498.70 bB	535.74 aA
	OMF	289.78 d	374.17 cA	455.11 bC	494.62 aB
	OCB	289.78 c	346.01 bB	551.78 aA	532.78 aA

Note: The lowercase letters indicate significant differences between fertilizing levels ($p<0.05$) within the same fertilizer type; the capital letters indicate significant differences between fertilizer types ($p<0.05$) within the same fertilizer level.

Table S6 Soil nitrogenase activities in all fertilization regimes under different sampling time points in the controlled experiment.

Sampling time (d)	Treatment	Nitrogenase activity (nmole $C_2H_4 \cdot g^{-1}$ dry soil$\cdot min^{-1}$)			
		Fertilizing level			
		None	Low	Medium	High
3	O	18.64 c	56.46 bA	74.13 aAB	78.03 aB
	OMF	18.64 c	23.59 cB	80.32 bA	94.74 aA
	OCB	18.64 c	21.86 cB	64.98 bB	94.29 aA
8	O	18.17 c	129.12 bB	130.15 abB	142.08 aAB
	OMF	18.17 c	167.51 aA	140.93 bAB	138.22 bB
	OCB	18.17 b	163.92 aA	154.06 aA	146.37 aA
16	O	17.86 d	134.80 aC	106.14 bB	83.87 cC
	OMF	17.86 d	190.01 aB	98.23 cB	124.75 bA
	OCB	17.86 c	224.75 aA	116.79 bA	106.82 bB
30	O	10.38 c	24.39 cC	222.71 aB	208.11 abB
	OMF	10.38 d	207.18 bB	240.21 aA	185.00 cC
	OCB	10.38 d	255.75 aA	182.17 cC	231.54 abA
45	O	12.30 b	25.02 aA	28.94 aAB	24.52 aA
	OMF	12.30 b	28.16 aA	25.78 aB	28.92 aA
	OCB	12.30 b	27.72 aA	33.18 aA	28.23 aA
60	O	13.17 c	25.77 aA	18.70 bB	24.98 aA
	OMF	13.17 b	14.88 bB	20.62 abAB	27.89 aA
	OCB	13.17 b	25.08 aA	24.30 aA	25.14 aA

Note: The lowercase letters indicate significant differences between fertilizing levels ($p<0.05$) within the same fertilizer type; the capital letters indicate significant differences between fertilizer types ($p<0.05$) within the same fertilizer level.

Table S7 The *P*-Values of factors fertilizer type, level, and their interaction effects on plant variables in the field experiment as analyzed by two-way ANOVA.

Plant variable	*P*-value		
	Fertilizer Type	**Fertilizer Level**	**Type × Level[1]**
Growth of height	0.001	<0.001	0.098
Growth of diameter	0.01	<0.001	0.295
Leaf biomass	<0.001	<0.001	<0.001
Transpiration rate	0.062	0.158	0.124
Stomatal conductance	0.012	0.107	0.093
Photosynthetic rate	0.025	0.044	0.033
Intercellular CO_2 concentration	0.014	<0.001	0.117
Carbon (C) centents in leaves	0.975	0.963	1
Nitrogen (N) centents in leaves	0.003	<0.001	<0.001
Phosphorus (P) centents in leaves	0.077	0.048	0.062
C/N ratio	0.56	0.049	0.711
C/P ratio	<0.001	<0.001	0.013
N/P ratio	0.725	0.422	0.867
Flavonoid concentration	0.039	0.022	<0.001
Triterpenoid concentration	0.129	0.145	0.118
Polysaccharide concentration	0.785	0.026	0.58
Flavonoid yield	0.034	0.05	0.003
Triterpenoid yield	0.033	0.038	0.047
Polysaccharide yield	0.486	0.005	0.478

[1] Type × Level = Fertilizer Type × Fertilizer Level interaction effect.

Chapter 6 Identifying soil microbial indicators relating the use of probiotic microbes to improved plant performance via a model-based approach

Adapted from: Wang Z, Chen Z, Leite Marcio FA, et al. Identifying soil microbial indicators relating the use of probiotic microbes to improved plant performance via a model-based approach. (Under Review)

Abstract

The soil microbiome impacts plant performance, but less is known about how to steer soil microbiome to modify plant metabolites by introducing probiotic consortia and how to identify microbial indicators for aboveground changes. Using *Cyclocarya paliurus* as a model medicinal plant and two mixtures of phyto-stimulatory and nutrient-enhancing probiotics delivered via bio-fertilizer, we applied generalized joint attribute model (GJAM) analysis to examine the impacts of bio-fertilizer level and probiotic consortia on soil microbiome assembly, plant nutrient stoichiometry and plant metabolic content over three successive years under field conditions. GJAM analysis showed that high fertilizer levels reduced the influence of the probiotic consortia on the whole system, with fewer differences observed between fertilizer types. Specific soil microbial taxa were identified as potential indicators of appropriate fertilization-inoculum combinations for optimal plant metabolite production, which link to leaf C: N: P stoichiometry. The fertilization-inoculum regimes predicted to be most effective were also validated in terms of plant metabolite production. The microbial indicators were further tested in pot experiment. This study shows that probiotic consortia can modulate plant metabolites by conditioning the soil microbiome and plant nutrient balance. The identification of microbial indicators provides a new perspective toward understanding below-aboveground interactions.

1. Introduction

Plant metabolites can exhibit a diverse range of biological activities, such as mitigation of environmental stress and plant defense against herbivores and soil-borne pathogens. Bioactive compounds extracted from the leaves, fruits or roots of medicinal plants are also a major source of natural pharmaceuticals for the development of medicinal drug products (Hussain et al. 2012). To improve the yield of bioactive compounds from medicinal plants, chemical fertilizers are widely used but have considerable negative impacts on the environment (Deng et al. 2019a). Recent research has revealed that plant growth and metabolite content are dependent on complex interactions with the biotic environment, including a close metabolic interplay with associated microorganisms in the rhizosphere, phyllosphere, and endosphere (Verhagen et al. 2004, Vorholt 2012). Thus, reshaping the biotic environment associated with the plant, such as soil microbiome, may be a pathway for improving plant metabolic performance, while reducing the use of chemical fertilizers (Xiong et al. 2017, Berg et al. 2020).

Microorganisms are key in reshaping plant performance. Plants and soil microorganisms share a long evolutionary history (van der Heijden et al. 2015), and this symbiotic relationship can increase the ecological adaptability of plants to environmental constraints (Selosse and Le Tacon 1998, Brundrett 2002, Ravanbakhsh et al. 2019b). On

the one hand, plants naturally assemble specific communities of microorganisms that colonize plant surfaces and the endosphere, leading not only to changes in plant metabolism, but also in the plant and soil microbiomes (van de Mortel et al. 2012, Huang et al. 2014, Etalo et al. 2018). On the other hand, such interactions can be more specifically exploited by using selected microorganisms isolated from the natural environment as biostimulants in plant-soil systems to enhance plant growth and crop quality (Armada et al. 2018). For instance, phyto-stimulatory strains like *Azospirillum* sp. and *Pseudomonas* sp. participate in synergistic metabolic activities with the host plant to increase the biosynthesis of certain secondary metabolites (Karthikeyan et al. 2009, Walker et al. 2012, del Rosario Cappellari et al. 2013). Other strains have been used to improve soil nutrient properties and the plant's internal nutrient balance, which important for modulating plant metabolism and metabolite production (Deng et al. 2019b, Liu et al. 2020b, Wang et al. 2021b). When added to poor soils, nutrient-enhancing strains like *Bacillus* sp. and *Azotobacter* sp. can influence nutrient acquisition and plant stoichiometry to improve plant growth (Saxena et al. 2013, Latef et al. 2020). This diversity of effects suggests that co-inoculating multiple microbial strains could provide an increased range of benefits to the host plant (Karthikeyan et al. 2009), but the combined effects of phyto-stimulatory and nutrient-enhancing strains remain poorly understood.

Microbial inoculants are increasingly used for soil bioremediation, biocontrol, and biofertilization, but the role of inoculant-induced soil microbiome in regulating plant performance has received limited attention (Vessey 2003, Xiong et al. 2017, Mawarda et al. 2020, Pagnani et al. 2020). Several studies have focused on the effects of bacterial inoculants on plant metabolites and the soil/plant microbiome (Schmidt et al. 2014), including the importance of the soil microbiota for metabolite accumulation in medicinal plants (Huang et al. 2018, Liu et al. 2020b). However, due to the multiple players and complex interactions involved, elucidating the precise mechanisms by which inoculated organisms elicit a specific plant phenotype remains challenging. Furthermore, incomplete analysis approaches that fail to integrate plant and soil parameters and other environmental factors with microbiome data may generate misleading and one-sided perspectives (B. Sohn and Li 2018, Leite and Kuramae 2020). In particular, our previous study (Leite and Kuramae 2020) and (Gloor et al. 2017) caution that the interactions between microbes and other variables should be represented within a model-based approach, as opposed to relying on simple correlation analyses. Because the compositional peculiarity of microbial community data biases correlations by inducing false significant relationships. To unravel the tripartite relationships among introduced beneficial microorganisms, the resident microbiome, and plant metabolic performance, an appropriate analysis method must be able to integrate all information and handle different sources of bias.

Cyclocarya paliurus (Batal.) Iljinsk, a woody medicinal plant belonging to the Juglandaceae family (Fang et al. 2011),

was selected as the model medicinal plant. Bioactive compounds extracted from *C. paliurus* leaves were mainly comprised of flavonoids, triterpenoids, and polysaccharides with antidiabetic, antioxidant, and antimicrobic effects (Fu et al. 2015, Wu et al. 2017). Co-inoculation of phyto-stimulatory and nutrient-improving microbial strains reshapes the root architecture of *C. paliurus* and induces shifts in plant nutrient balance and phytochemical accumulation (Wang et al. 2021b). However, the impact of successive introduction of microbial consortia on the resident microbiome and the role of the soil microbiota in regulating host performance, especially metabolite production, are not well understood.

In this study, we hypothesized that introduced probiotic consortia could change the plant metabolites and growth performance by directly affecting the plant nutrient stoichiometry and indirectly conditioning the resident soil microbiome. To test these hypotheses, a field experiment was conducted on a *C. paliurus* plantation for three successive years to assess the effects of co-inoculating phyto-stimulatory and nutrient-improving probiotics in combination with bio-organic fertilizer at three different doses. In addition, a pot experiment was conducted to test the potential microbial indicators and identify those that were responsive in both the field and pot experiments. The aims of the present study were threefold: (i) to evaluate the impacts of probiotic consortia on plant growth performance and metabolite production under degraded field conditions (low-fertility), (ii) to investigate the responses of the resident soil microbiome to different fertilization and inoculation regimes, and (iii) to integrate above- and belowground parameters using a generalized joint attribute model approach to identify the optimal bio-fertilization regime for maximal plant bioactive compound yield and predict the key soil taxa that might function as microbial indicators.

2. Materials and Methods

Plant probiotics and organic fertilizer

Two nutrient-enhancing strains (C: *Azotobacter chroococcum* HKN-5, M: *Bacillus megaterium* W17) and two phyto-stimulatory strains (B: *Azospirillum brasilence* CW903, F: *Pseudomonas fluorescens* W12) were utilized in our study; M was paired with F, and C was paired with B (see Figure 6.1). These strains have been shown to improve soil nutrient availability and plant growth and do not exhibit antagonistic effects against each other (Wang et al. 2019c, Wang et al. 2021a, Wang et al. 2021b). The preparation of each inoculant was as described previously (Wang et al. 2021b). Briefly, each strain was grown in lysogeny broth medium until the logarithmic growth phase. Bacterial suspensions were adjusted to a final concentration of 1×10^8 colony-forming units (CFU)·mL^{-1} for each strain. The

organic fertilizer was mainly comprised of chicken manure, straw, tea dross and mushroom dross; the chemical properties of the fertilizer material are given in Table S1.

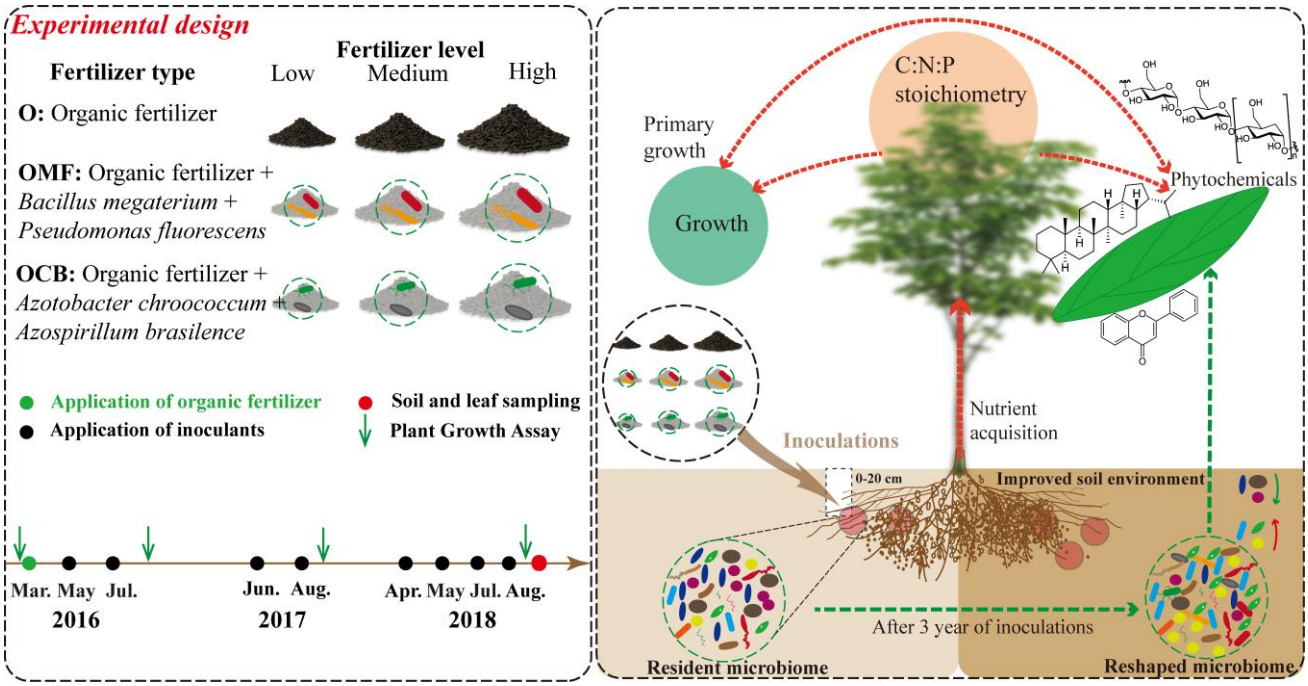


Figure 6.1 Experimental design, timeline of fertilization and sampling, and conceptual outline of the study. Fertilizer level: Low = 10^7 cells + 0.5 kg organic fertilizer per plant; Medium = 10^8 cells + 1.0 kg organic fertilizer per plant; High = 10^9 cells + 1.5 kg organic fertilizer per plant.

Experimental design

The plantation site is in typical subtropical zone with soil is classified as a yellowish-brown clay soil with low-fertility. The field experiment site and soil properties have been described in detail in a previous study (Wang et al. 2021b). The experiment was laid out in a three-block pattern based on a completely randomized factorial design with three treatment types (O: organic fertilizer; OCB: organic fertilizer applied with *A. chroococcum* and *A. brasilence*; OMF: organic fertilizer applied with *B. megaterium* and *P. fluorescens*) and three fertilization levels (low, medium, high: 10^7, 10^8, 10^9 cells and 0.5, 1.0, 1.5 kg organic fertilizer per plant), giving a total of nine fertilization regimes (O1, O2, O3, OCB1, OCB2, OCB3, OMF1, OMF2, OMF3) and one control (non-fertilized) in the field. Detailed information is provided in Table S2 and Figure 6.1.

Each treatment contained at least 60 *C. paliurus* seedlings that were equally divided into three blocks. All organic fertilizers were applied in the field in March 2016, while inoculations were conducted over a period of three years in May and July 2016, June and August 2017, and April, May, July, and August 2018. An interval of 45 days between

inoculations in the same year was selected based on bacterial growth curve data and the effects of the inoculations on soil properties (Wang et al. 2019c). Before the first fertilizer application, a 20-cm-deep circle was dug around the plant's vertical canopy projection as the fertilizer zone to provide better access to the lateral roots. The same procedure was carried out for the control plants without fertilization. The resident microbiome in the non-fertilized soil collected at the end of the experiment is presented in Figure S1.

Plant growth measurements

Plant growth was evaluated by measuring seedling height and stem basal diameter of all healthy seedlings (20 plants from each treatment in each block) in March 2016 and September 2018. Net growth in terms of height or stem basal diameter was calculated as the difference between the initial and final measured values.

Soil and leaf sampling

In September 2018, soils were vertically sampled at a depth of 0-20 cm in the fertilizer zone using the hole-sampling method (Figure 6. 1). Briefly, in each block, five vertical holes (diameter: 5 cm; depth: 20 cm) were made using a sampling tube for the same three seedlings that were selected for leaf sampling in each treatment. After removing plant material, stones and other debris, the soil samples from one block were pooled equally and mixed thoroughly to form a composite sample. This resulted in three composite samples for each treatment. A portion of each sample was stored at 4 °C until the analysis of biochemical properties, and another portion was stored at -20 °C prior to DNA extraction. Fresh leaf material was also harvested in September 2018, with nine replicates for each treatment. All samples were dried at 60 °C, ground into powder and stored at room temperature until measurement of nutrients and bioactive compounds.

C:N:P stoichiometry in leaves

Total C and N contents were determined by an elemental analyzer (vario MAX CN, Elementar, Hanau, Germany) using 50.0-mg leaf material wrapped in aluminum foil. Total P was measured by the molybdenum-blue method by digesting a 1-g sample with HNO_3 and $HClO_4$ (5:1 in volume). The C/N, C/P and N/P ratios were then calculated.

Bioactive compounds in leaves

Flavonoids were extracted from *C. paliurus* leaves using an ultrasonic-assisted method with 75% ethanol after removing fat-soluble impurities with petroleum ether. The total flavonoid concentration was determined using a colorimetric method with detection at 415 nm (Bao et al. 2005), referenced to a standard Rutin curve and expressed as milligrams Rutin equivalent per gram of dry mass (mg/g). Water-soluble polysaccharides were extracted from *C. paliurus* leaves as described previously by Fu et al. (2015), and polysaccharide concentrations were determined by the phenol-sulfuric acid method. For triterpenoid extraction, 2.0 g of leaf material was extracted using an ultrasonic-assisted method. Briefly, 50 mL of 75% ethanol was added to each sample, and the extraction was conducted for 45 min at 65 °C and repeated twice. The total triterpenoid concentration was determined according to a previously described colorimetric method (Fan and He 2006). The total yields of these bioactive components in leaves were calculated by multiplying the concentration by the biomass of leaves.

Soil DNA extraction, PCR and Illumina MiSeq sequencing

Total soil DNA was extracted using the NucleoSpin® Soil DNA Kit (Macherey-Nagel GmbH & Co.KG, Düren, Germany), according to the manufacturer's protocol. Final DNA concentrations and purity were determined by a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis. Detailed information concerning PCR conditions and Illumina MiSeq sequencing is provided in Supplementary Methods.

Sequence data processing

Raw DNA sequences were processed with the DADA2 (Divisive Amplicon Denoising Algorithm 2) pipeline (Callahan et al. 2016) using the "dada2" package (v.1.7) in R 4.0.1 (R Core Team 2019). DADA2 infers true biological sequences of reads (i.e. amplicon sequence variants [ASVs]) from Illumina sequence data and does not involve sequence clustering (Callahan et al. 2017). Briefly, primers and adapters were screened and removed using the "cutadapt" package (v.2.10). Raw sequences were first demultiplexed by comparing index reads with a key and then quality-filtered by using the filterAndTrim function. Error rates were subsequently derived from a set of subsampled reads (i.e. 1 million random reads). Sequences were dereplicated, and unique sequence pairs were denoised using the "dada" function. Finally, paired-end sequences were merged, and chimeras were removed. Taxonomy group information was then assigned using the SILVA databases (v.138) (Quast et al. 2013). Raw sequences of the field

experiment were submitted to the NCBI Sequence Read Archive (SRA) under the accession number from SRR13756033 to SRR13756062 in a BioProject PRJNA703386: https://dataview.ncbi.nlm.nih.gov/object/PRJNA703386?reviewer=jg9035qgj2srrq1dglr5caft0f. Raw sequences of the pot experiment were submitted to SRA in a BioProject (PRJNA630558).

Data analysis

Two-way ANOVA was used to estimate the effects of the different fertilization regimes on plant growth and nutrient acquisition, and Duncan's test was used to compare the effects between treatments. The median concentrations and yields of bioactive compounds in *C. paliurus* leaves were calculated within each fertilizer type to compare the differences between fertilizer levels and within each fertilizer level to compare the differences between fertilizer types. Polynomial regression analysis was used to examine the relationship between bioactive compounds and C/N and C/P ratios, and the results were plotted by the ggplot function in the "ggplot2" package. R-square values were used for evaluate the fitness of the regression curve, and the F-test was applied to test if a polynomial model provided a significantly better fit than the intercept-only model.

To analyze the responses of soil bacterial alpha diversity to different fertilization regimes, "observed", "Shannon", "InvSimpson", and "Fisher" were selected and plotted by the plot_richness function in the "phyloseq" package (McMurdie and Holmes 2013). Beta diversity was visualized by non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis distance, which was calculated by the ordinate function in the "phyloseq" package. The resident microbiome in soil that did not receive any fertilizer was analyzed after Centered Log-Transformation (CLR). To examine the community composition under the different fertilization regimes, the rare taxa were first identified by the aggregate_rare function in the "microbiome" package, and then the whole community composition was plotted by using the plot_composition function in the "phyloseq" package. Linear discriminant analysis (LDA) was subsequently applied to identify potential bioindicator taxa and the effects of different fertilization regimes on those taxa.

To examine the impacts of the different fertilization regimes on the whole plant-soil system and to identify potential underlying plant, soil and microbial contributors to the observed patterns, we carried out generalized joint attribute model (GJAM) analysis in the "gjam" package (v.2.3.2) developed by Clark et al. (2017). Detailed information concerning model construction is provided in the Supplementary Methods.

Multiple correspondence analysis (MCA) was applied to examine how the different fertilization regimes affected the whole system and to identify the top variables contributing to the variance by using the dataframe from the

model list. As a supplement, a Sankey plot was created using the SankeyDiagram function in the “flipPlots” package based on the coefficients to assess how fertilizer type and level affected the plant and soil variables and microbial communities in the system. To estimate the effects of different fertilization regimes on the top 10 microbial contributors in the system, we compared the coefficients between different fertilizer types and levels, and the results were visualized using the ggplot function. A GJAM model was further used to identify the key microbial indicators for appropriate fertilization regimes and improved plant metabolite production. More detailed information describing this method has been provided in the Supplementary Method.

Additional pot experiment

To complement our field experiment, we collected soil from the field and conducted a pot experiment using the same fertilizer and strains to compare potential microbial indicators and identify common indicators that were responsive in both the field and pot experiments. Detailed information concerning the design of this pot experiment is provided in Figure S2.

3. Results

Plant growth and nutrient acquisition in leaves

Three levels of bio-fertilizer were assessed in the field experiment: low, 10^7 cells and 0.5 g of organic fertilizer per plant, medium, 10^8 cells and 1.0 kg of organic fertilizer per plant; and high, 10^9 cells and 1.5 kg of organic fertilizer per plant. As shown in Table S3, bio-fertilization increased net plant growth in terms of height and stem basal diameter, but this effect was highly variable across the different fertilizer types and levels. For instance, O2 (O at medium fertilizer level), OCB2 (OCB at medium fertilizer level) and OMF2 (OMF at medium fertilizer level) all significantly improved net growth as measured by plant height compared to the control (no fertilizer), but OCB2 and OMF2 exhibited significant advantages over the application of organic fertilizer alone ($p<0.05$). Most fertilization regimes increased net growth as measured by basal diameter, but OCB2 and OMF2 had the largest effects ($p<0.05$). Similarly, application of the different fertilizers improved plant nutrient status compared with the non-fertilized control ($p<0.05$, Table S3). Specifically, treatments OCB2 and OMF2 increased leaf nitrogen (N) content compared with the control, and most of the fertilization regimes increased leaf phosphorus (P) content. As a result, changes in the C/N and C/P ratios were also observed, with lower ratios in the treatments with higher leaf contents of N and P, such as OCB2, OCB3, and OMF2. No significant effects of the treatments were found for

N/P ratios.

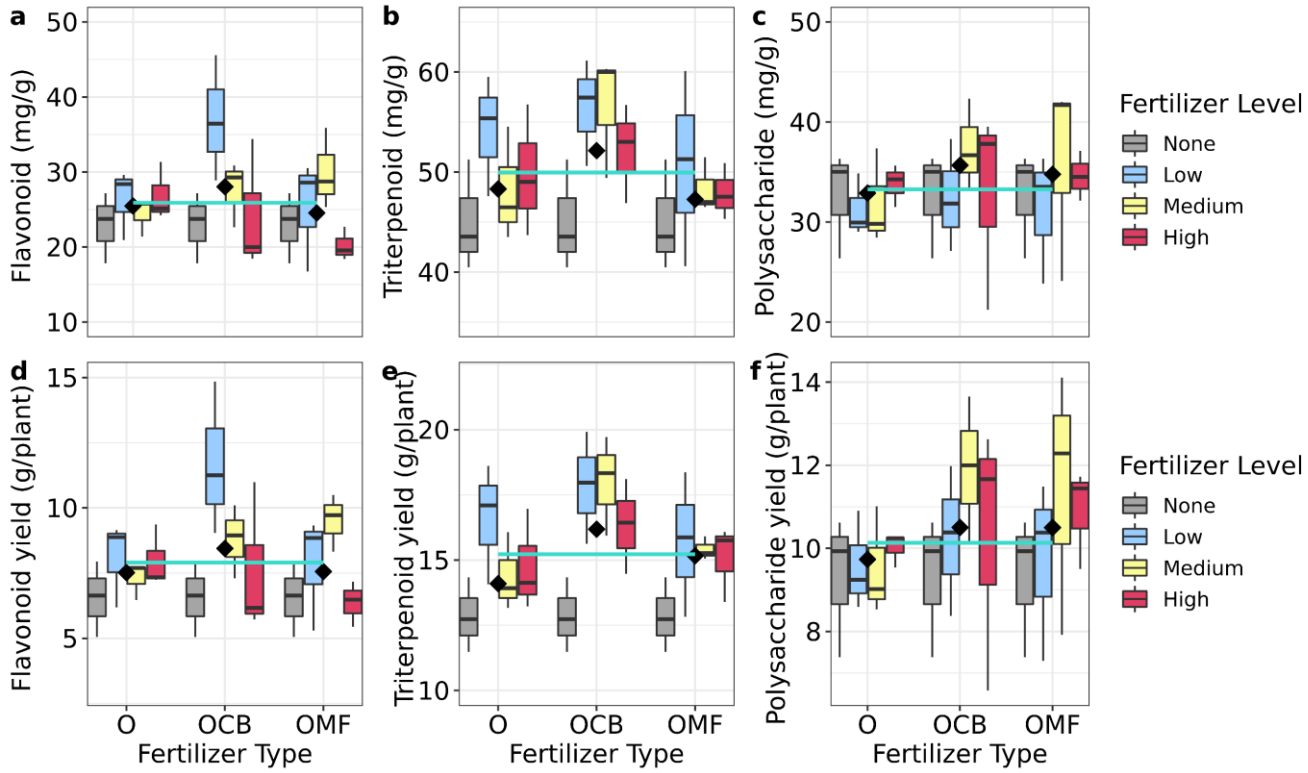


Figure 6.2 Effects of different fertilization regimes (type and level) on the total concentrations of flavonoids (a), triterpenoids (b), and polysaccharides (c) and on the total yield of each metabolite (d, e, f). The black rhombus indicates the median value for each fertilizer type and serves as a reference for comparing the variation among different fertilizer levels in the same fertilizer type. The blue line was calculated by using treatment O as a reference group to compare the variation among different fertilizer types. Fertilizer type: O = only organic fertilizer, OMF = organic fertilizer and inoculant containing both *Bacillus megaterium* and *Pseudomonas fluorescens*, OCB = organic fertilizer and inoculant containing both *Azotobacter chroococcum* and *Azospirillum brasilense.* Fertilizer level: Low = 10^7 cells + 0.5 kg organic fertilizer per plant, Medium = 10^8 cells + 1.0 kg organic fertilizer per plant, High = 10^9 cells + 1.5 kg organic fertilizer per plant.

Accumulation of bioactive compounds and relationships with nutrient stoichiometry

Both the type and level of fertilizer influenced the accumulation of total flavonoids, triterpenoids, and polysaccharides in *C. paliurus* leaves, as shown in Figure 6.2. Compared with organic fertilization alone (O), the introduction of beneficial bacteria at specific fertilizer levels increased both the concentration and yield of flavonoids and triterpenoids (Figure 6.2a, b, d, e). For instance, treatments OCB1 and OMF2 increased flavonoid levels, while treatments OCB1 and OCB2 enhanced the accumulation of triterpenoids. However, at the highest

fertilizer level among the OMF treatments (OMF3), the concentration and yield of flavonoids decreased significantly. No significant effects of the treatments on polysaccharide production were observed.

To visualize the relationship between leaf nutrient stoichiometry and the accumulation of bioactive compounds, scatter plots of the C/N and C/P ratios versus the concentration and yield of bioactive compounds are shown in Figure 6.3. Both flavonoids and triterpenoids exhibited strong correlations with the C/N and C/P ratios, and production was highest at medium C/N (approximately 24) and C/P (approximately 280) ratios (Figure 6.3). However, no clear patterns were observed with respect to relating polysaccharide accumulation with *C. paliurus* leaf nutrient stoichiometry, nor were there significant correlations between the N/P ratio and bioactive compounds.

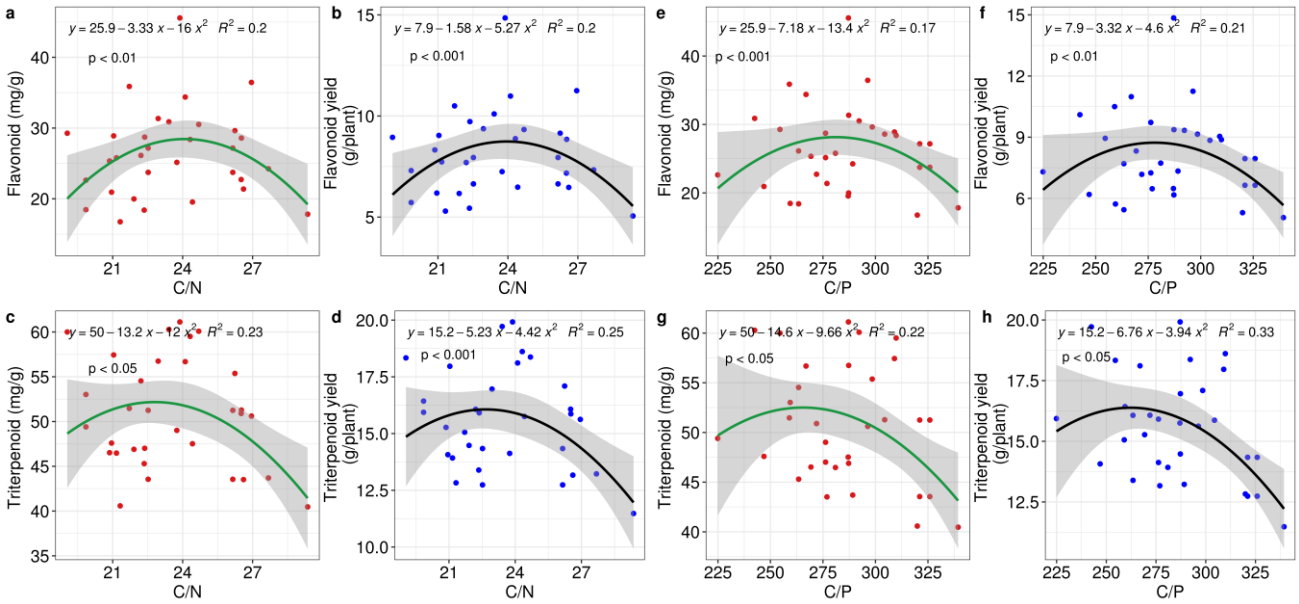


Figure 6.3 The relationships between the C/N ratio (a-d) or the C/P ratio (e-f) with the concentration (red points) and yield (blue points) of bioactive compounds.

Responses of the resident microbial community to different fertilization regimes

After filtering and trimming of the sequence data, a total of 8796 denoised amplicon sequence variants (ASVs) were recovered from the 30 experimental samples. The application of bio-fertilizer enhanced bacterial alpha diversity (Figure S3), and the differences among the three fertilizer types increased with the fertilizer level (Figure S3a). Similarly, both fertilizer type and level affected the dissimilarities in the soil bacterial community (Figure S3b) and the abundances of specific phyla (Figure S3c). Treatments OCB2, OCB3, OMF2, and OMF3 increased the abundances of the top 10 phyla compared with the other treatments ($p<0.05$). Linear discriminant analysis (LDA)

confirmed that treatments OCB2 and OCB3 increased the abundance of *Actinobacteriota* and *Gemmatimonadota*, treatment OMF2 increased the abundance of *Proteobacteria*, and treatment OMF3 increased the abundance of *Chloroflexi* (Figure S4).

Effects of fertilization regime on the whole system and the underlying plant, soil and microbial contributors

As shown by the multiple correspondence analysis (MCA) plot (Figure 6.4a), the influence of fertilization on the whole system was highly dependent on fertilizer level and type. When the fertilizer level was low, the introduction of beneficial microbes had a large impact compared with the application of organic fertilizer only (O). However, at higher fertilizer levels, the impact of microbial introduction was less pronounced (Figure 6.4a). The Sankey plot shows how fertilizer type and level affected the plant and soil variables and microbial communities in the studied system (Figure 6.4c). The impacts of OCB and OMF on the microbial community were larger than those of O, but decreased with increasing fertilizer level.

We identified the top 10 variables derived from model analysis of the whole system (Figure 6.4b). Plant and soil variables appeared to be more important than microbiome features in influencing the whole system. Soil inorganic N and soil available P explained the most variance along axis 1, and plant height and survival rate explained the most variance along axis 2. The top 3 microbial taxa were the genus *Pseudomonas* and the orders *Acidobacteriales* and *Acidobacteriota* Subgroup 2. Furthermore, in this model, we filtered the top 10 microbial taxa contributing to whole-system variance and evaluated the effects of different fertilization regimes on these taxa (Figure S5). As the fertilizer level increased, the three fertilizer types exerted different effects: O and OCB enhanced the taxa *Acidobacteriales* and *Acidobacteriota* Subgroup 2, whereas OMF decreased these taxa.

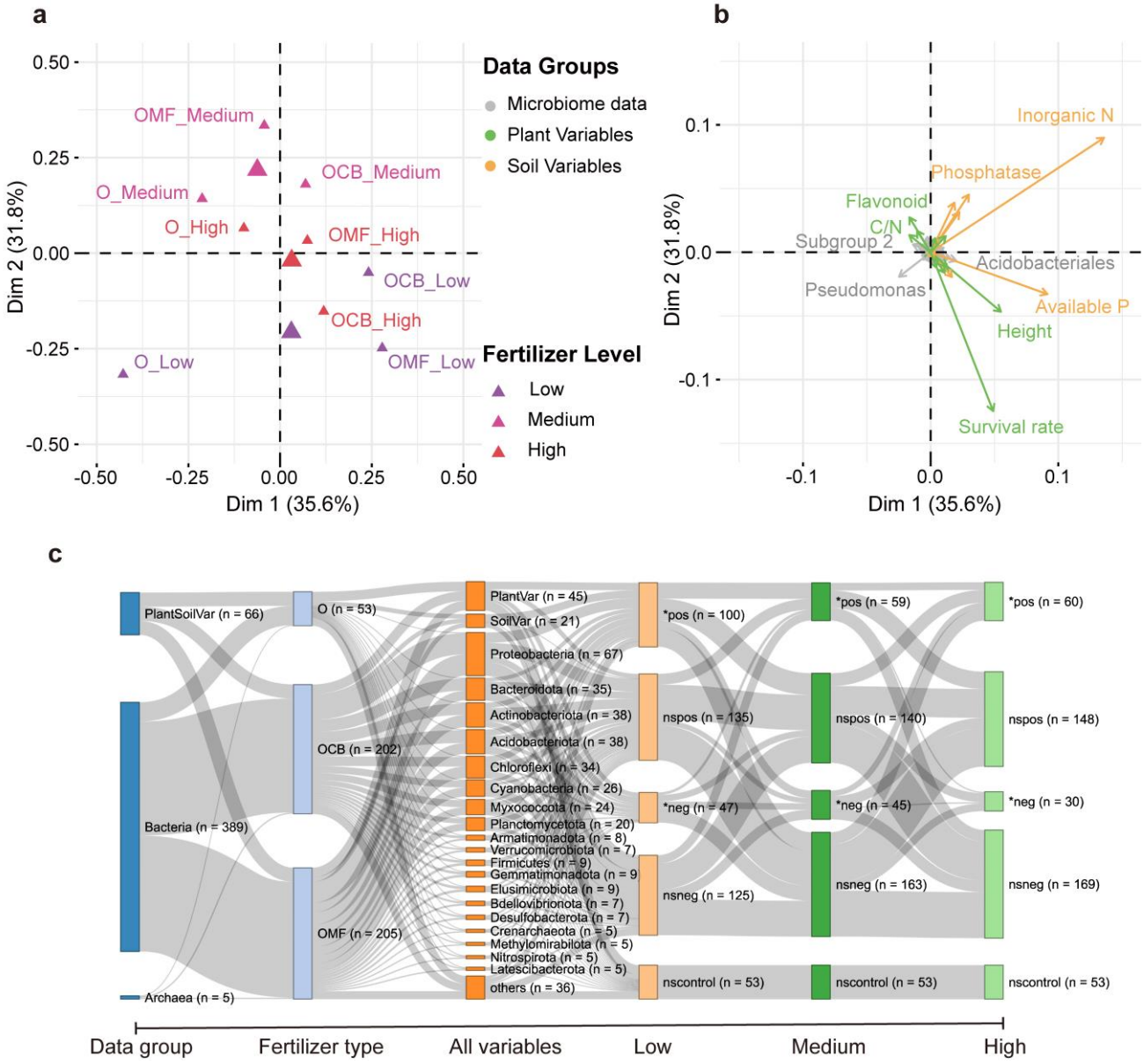


Figure 6.4 Differentiation of fertilization regimes and underlying plant, soil and microbial contributors by generalized joint attribute modeling (GJAM). (a) Multiple correspondence analysis (MCA) plot of all fertilization regimes. (b) Identification of the top 10 contributors in the whole system. (c) Within the same fertilizer level, the differences among the fertilizer types were examined by comparison against the reference treatment O. PlantSoilVar: plant and soil variables; *pos: the effects were significantly positive; nspos: the effects were non-significantly positive; *neg: the effects were significantly negative; nsneg: the effects were non-significantly negative; nscontrol: the reference (treatment O) used in comparisons with other treatments. Fertilizer type: O = only organic fertilizer; OMF = organic fertilizer and inoculant containing both *Bacillus megaterium* and *Pseudomonas fluorescens*; OCB = organic fertilizer and inoculant containing both *Azotobacter chroococcum* and *Azospirillum brasilense.* Fertilizer level: Low = 10^7 cells + 0.5 kg organic fertilizer per plant; Medium = 10^8 cells + 1.0

kg organic fertilizer per plant; High = 10^9 cells + 1.5 kg organic fertilizer per plant.

Identifying soil microbial indicators linked to the accumulation of bioactive compounds under different fertilization regimes

To evaluate the effects of different fertilization regimes on the accumulation of bioactive compounds, we analyzed (1) microbial taxa linked to flavonoid accumulation using predictions generated by our generalized joint attribute model (GJAM) analysis (Figure 6.5a) and (2) appropriate combinations of fertilizer level and type to achieve the maximum flavonoid concentration (Figure 6.5b-d). As shown in Figure 6.5a, we predicted microbial abundances producing the maximum concentration of flavonoids and compared the predicted and observed values. The predicted abundances of microbes belonging to the phylum *Proteobacteria* were higher than the observed values, indicating that increasing the abundances of such microbes might facilitate the accumulation of flavonoids. The opposite pattern was observed for microbes belonging to the phyla *Actinobacteriota* and *Chloroflexi*. Similarly, the predicted and observed effects of the fertilization regimes on microbes associated with flavonoid accumulation are compared in Figure 6.5b. The mean values of the predicted microbial abundances were higher than the observed values for treatments OCB1 and OMF2, whereas the opposite pattern was observed for treatment OMF3. These patterns are in accordance with the observed variation of flavonoid concentration and yield (Figure 6.2) Furthermore, the predicted and observed values of microbial abundance were compared to determine the top 10 microbial indicators that should putatively be increased (Figure 6.5c) and the top 10 that should putatively be decreased (Figure 6.5d) to achieve the highest production of flavonoids. At the lowest level of fertilization, O decreased the abundance of most of the desirable taxa compared with OCB and OMF (Figure 6.5c), whereas the opposite pattern was observed at the highest level of fertilization. By contrast, at the lowest level of fertilization, OMF and OCB decreased the abundances of the least-favorable taxa compared with O, while few differences among these treatments were observed at the medium and high fertilization levels. Similar patterns were observed for triterpenoids (Figure S6): the abundances of most of the desirable taxa for triterpenoid accumulation were higher under OCB than the other fertilizer types at low and medium levels of fertilization, and both OCB and OMF decreased the abundances of the top 10 least-favorable taxa for triterpenoid accumulation.

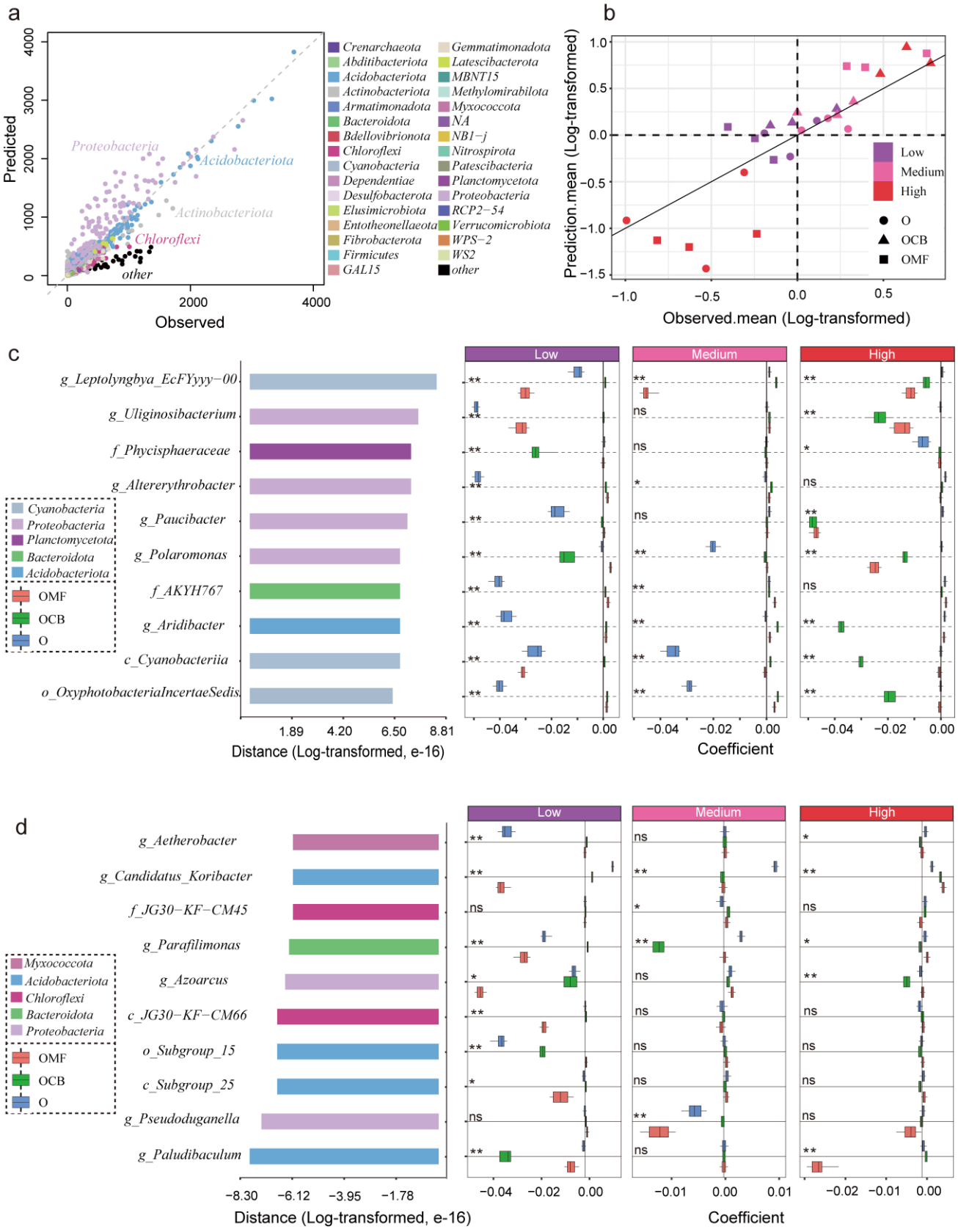


Figure 6.5 Prediction of optimal flavonoid accumulation based on microbiome data in association with different fertilization regimes in GJAM. (a) Predicted and observed abundances of all phyla in stimulating the accumulation

of flavonoids. To achieve the highest accumulation of flavonoids in this model, the abundance of the microbes above the dashed line should be increased, while the abundance of the microbes below the dashed line should be decreased. (b) Effects of all tested fertilization regimes on the accumulation of flavonoids with attribution of microbes. The treatments above the solid line resulted in a positive effect on the accumulation of flavonoids. (c and d) Identification of the top 10 microbial taxa (c_: class; O_: order; g_ : genus; f_: family) that improved (c) or hampered (d) the accumulation of flavonoids and their responses to the different fertilization regimes.

Comparison of field and pot experiments for potential microbial indicators of high flavonoid accumulation

To further examine the potential microbiota associated with high accumulation of flavonoids under different conditions, we compared field and pot experiments to identify common potential indicators. In total, 149 and 168 positive potential candidates (microbes that should be increased to achieve high flavonoid accumulation) were identified in the field and pot experiments, respectively (Figure 6.6a). A total of 36 potential candidates distributed over 9 phyla were held in common between the two experiments: *Proteobacteria* (36%), *Bacteroidota* (14%), *Myxococcota* (14%), *Chloroflexi* (11%), *Acidobacteriota* (11%), *WS2* (2.7%), *Armatimonadota* (2.7%), *Cyanobacteria* (2.7%), and *Firmicutes* (2.7%). Although the magnitude of the impact of many of these candidates differed between the two experiments, the influence of others was nearly identical, including *g_Rhodoplanes*, *g_Stenotrophobacter*, *g_Bryobacter*, and *g_Pajaroellobacter*. We also identified 139 and 140 negative potential candidates (microbes that should putatively be decreased to improve flavonoid accumulation) in the field and pot experiments (Figure 6.6b), respectively. Of these candidates, 29 belonging to 10 phyla were held in common across the two experiments: *Proteobacteria* (41%), *Chloroflexi* (20%), *Acidobacteriota* (7%), *Armatimonadota* (7%), *Planctomycetota* (7%), *Verrucomicrobiota* (3.5%), *Bacteroidota* (3.5%), *Gemmatimonadota* (3.5%), *Latescibacterota* (3.5%), and *Myxococcota* (3.5%). The magnitude of the impact of candidates like *g_Steroidobacter*, *g_Opitutus*, *f_Fimbriimonadaceae*, and *p_Latescibacterota* on flavonoid accumulation was nearly identical between the two experimental systems.

The potential role of these microbial indicators in association with plant metabolic performance and the effects of probiotic addition on the relative abundance of microbial indicators were tested in the pot experiment (Figure 6.7). Comparing to single application of organic fertilizer (O), the introduction of probiotic

consortia (OCB, OMF) significantly increased the relative abundance of these positive candidates and significantly decreased the relative abundance of negative candidates (Figure 6.7a, b). Hence, microbial indicators could assist or impede the beneficial effects of probiotics on plant metabolites. Confirming this result, the flavonoids concentrations were significantly enhanced in the treatments OCB and OMF, but no significant difference was observed between OCB and OMF by ANOVA test (Figure 6.7c).

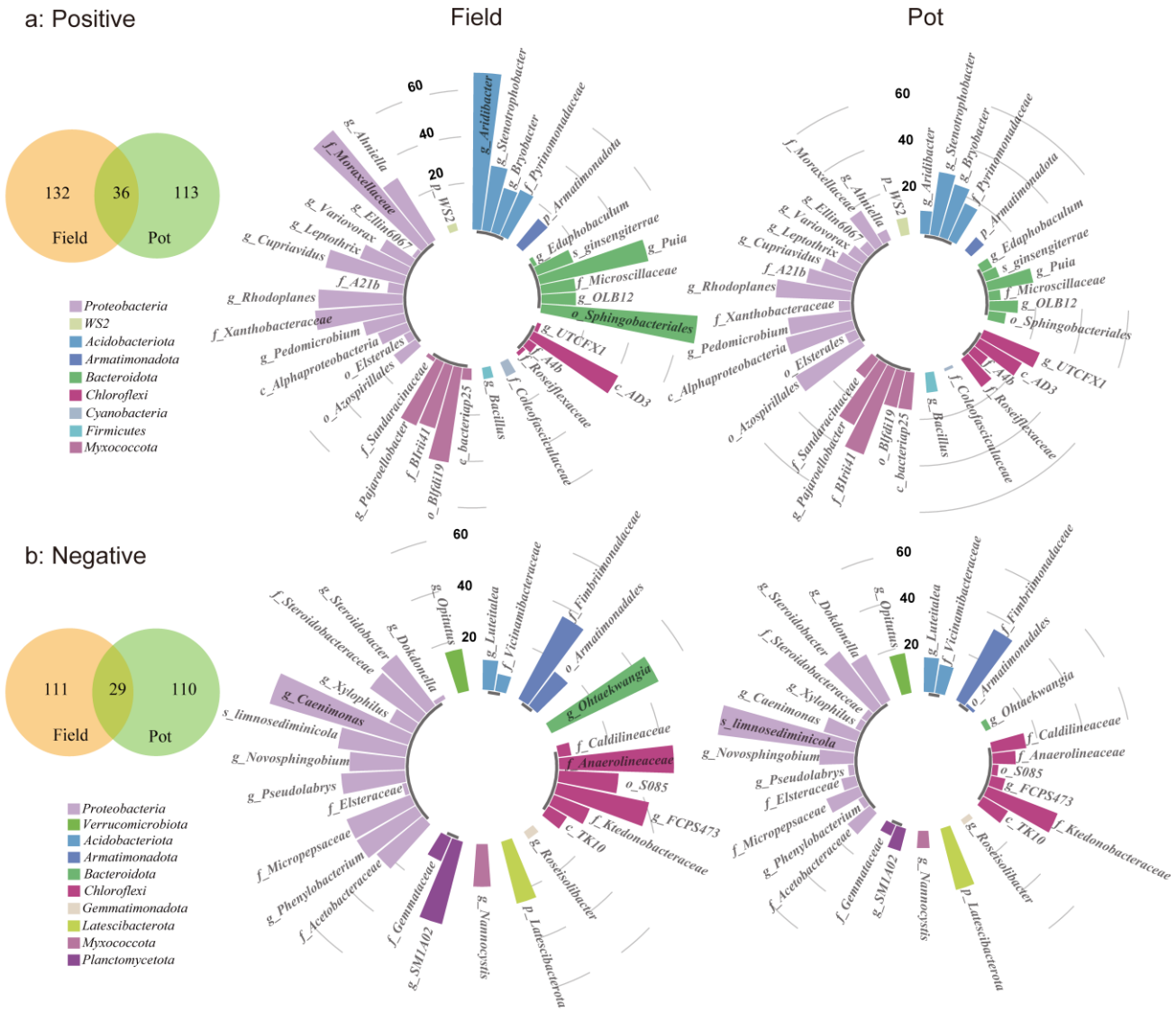


Figure 6.6 Identification of the common potential microbial indicators in field and pot experiments for improving the accumulation of plant flavonoids. (a) Positive common microbial indicators for stimulating the accumulation of flavonoids and their contributions in the field and pot experiments. (b) Negative common microbial indicators for stimulating the accumulation of flavonoids and their contributions in the field and pot experiments.

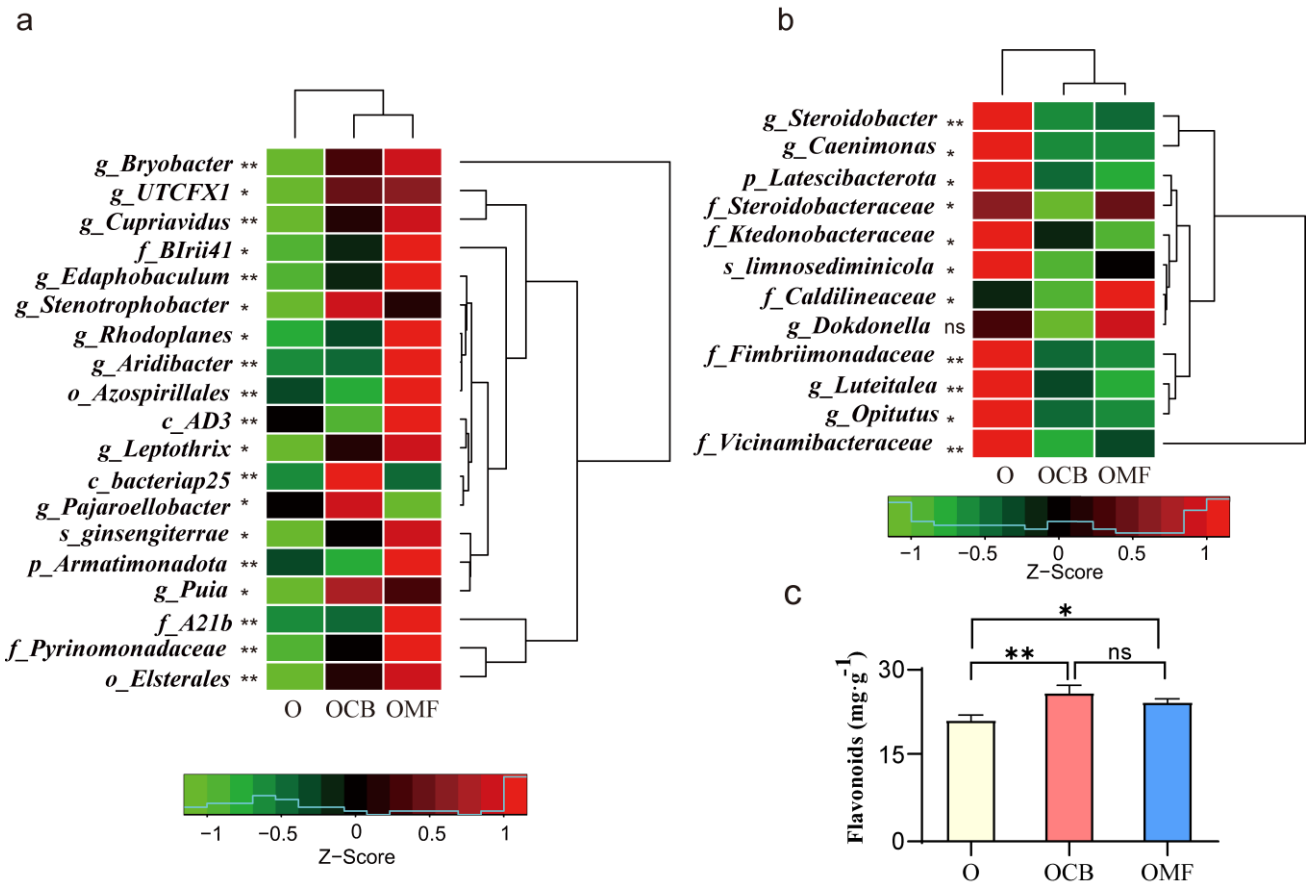


Figure 6.7 Impacts of probiotic consortia on the relative abundance of microbial indicators (indicators that have statistically significance) relating to the accumulation of flavonoids in pot experiment. (**a**) The relative abundance of positive microbial indicators in treatments O, OCB and OMF. (**b**) The relative abundance of negative microbial indicators in treatments O, OCB and OMF. (**c**) The effects of different treatments on the concentrations of flavonoids in *C. paliurus*.

4. Discussion

Effects of bio-fertilization on plant growth, nutrient acquisition, and the accumulation of bioactive compounds

Co-inoculation of multiple beneficial microbes has been suggested as an efficient means of stimulating host plant growth and protecting plants against environmental stress (Walker et al. 2012, Wang et al. 2016). Furthermore, combining co-inoculation with organic fertilization enables synergistic interactions that stimulate physical or biochemical activities while simultaneously improving microbial viability (Tejada et al. 2008, Yu et al. 2012). The

aim of the present study was to assess the integrated effects of combined application of phyto-stimulatory and nutrient-enhancing bacterial strains and organic fertilizer on *C. paliurus* growth, nutrient acquisition and accumulation of bioactive compounds compared with the application of organic fertilizer alone. After three years of bio-fertilization, application of the medium level of fertilizer with beneficial microbes best stimulated the growth of *C. paliurus*. The plant growth-promoting effects of organic fertilizer are mainly attributable to the high amount of organic matter, which includes N-rich materials and gradually extractable nutrients that can help improve soil fertility (Mitchell and Tu 2006). However, only a small fraction of the fertilizer applied to soil is assimilated by plants, with nutrient losses often in the range of 60%-90% (Adesemoye and Kloepper 2009). Previous studies of wheat (*Triticum aestivum* L.) (Nosratabad et al. 2017), sunflower (Arif et al. 2017), and soybean (Moretti et al. 2020b) have indicated that the introduction of beneficial inoculants can increase fertilizer-use efficiency. In the present study, higher contents of N and P were observed in *C. paliurus* leaves in the treatments that received beneficial microbes. Thus, the combined application of inoculants and organic fertilizer improved the fertilizer-use efficiency, leading to increased nutrient acquisition and plant growth promotion.

To increase the medicinal value of *C. paliurus*, appropriate plantation management strategies are needed that enhance the yield of target bioactive compounds in the leaves. Accumulating evidence suggests that the biosynthesis of bioactive compounds in *C. paliurus* leaves is influenced not only by the genetic background of the plant, but also by the cultivation practices employed, such as chemical fertilization, control of light quality, and availability of shade (Fu et al. 2015, Liu et al. 2018b, Deng et al. 2019a). However, information on the effects of introducing beneficial microbes on the metabolite content of *C. paliurus* leaves is limited. In the present study, flavonoid and triterpenoid production were enhanced by co-inoculating phyto-stimulatory and nutrient-enhancing bacterial strains at low (10^7 cells per plant) and medium (10^8 cells per plant) levels of fertilizer application. The optimal densities of beneficial microbes to achieve plant growth-promotion effects and biological productivity may differ depending on the specific microbe. For instance, 10^6-10^7 cells of *A. brasilense* per wheat plant were required to obtain a positive effect on the plant in previous work, whereas 10^5-10^6 cells per plant was suitable for *Pseudomonas* sp. application (Bashan 1986, Haas and Defago 2005). The influence of organic fertilizer on the C/N ratio in the soil may also impact microbial activities and plant metabolism (Deng et al. 2019b, Jasso-Flores et al. 2020).

In addition to the effect of fertilizer level, a strong relationship between leaf nutrient stoichiometry and bioactive compound accumulation was observed in our study: medium values of the C/N (approximately 24) and C/P (approximately 280) ratios were associated with the highest production of flavonoids and triterpenoids. Effects of

the internal nutrient balance on plant secondary metabolite production have been reported previously (Lillo et al. 2008). C, N and P are central to nearly all biochemical pathways in plants, so changes in internal nutrient stoichiometry (C/N, C/P) influence both primary growth and secondary metabolite production (Gigolashvili and Kopriva 2014, Canovas et al. 2018). Our findings are in partial agreement with the predictions of the growth-differentiation balance (GDB) hypothesis (Stamp 2003, 2004), which states that plants allocate more resources to secondary metabolism when experiencing an intermediate resource level. However, it should be noted that not all treatments that produced medium C/N and C/P levels resulted in the highest accumulation of metabolites, and thus other factors, such as plant-associated microorganisms, may also play important roles in regulating plant metabolism (Badri et al. 2013, Etalo et al. 2018).

Potential impact of soil microbiome changes on the accumulation of bioactive compounds in response to different fertilization regimes

Resident soil microbial communities are frequently subjected to (a)biotic disturbances due to agricultural management practices (fertilization and application of biocontrol and microbial inoculants) as well as naturally occurring disturbances (drought, flooding, and frost) (van Elsas et al. 2012, Mallon et al. 2015). The addition of microbes to the soil, whether beneficial inoculants for promoting plant productivity or pathogens affecting plant health, can alter the native microbial community by causing resource competition, antagonism, and synergism (Hu et al. 2016, Wei et al. 2018b, Mawarda et al. 2020). In contrast to previous studies limited to only microbial parameters, here we used GJAM analysis to build a full plant-soil system model incorporating multiple variables to examine the impacts of different fertilization regimes on the whole system and to identify potential underlying plant, soil and microbial drivers. Both MCA and Sankey plots showed that elevated fertilizer levels decreased the influence of bio-fertilization on the whole system, thereby reducing the differences in the effects of the three types of treatments. High amounts of fertilizer would be expected to elicit large changes in the microbial community, and nutrient-induced shifts in copiotrophic vs. oligotrophic microbial lifestyles could also affect soil functioning and plant performance (Wieder et al. 2013, Leff et al. 2015). Previously developed experimental frameworks have suggested that soil microbes are preferentially recruited by host plants under (a)biotic stress in plant-soil systems, whereas an improved soil environment for the host plant leads to lower dependence on beneficial microbes from the soil (Rashid et al. 2016, da Costa et al. 2020, Liu et al. 2020a). Similarly, our study found that the effects of

inoculation with beneficial microbes decreased at higher fertilizer levels.

The interplay between the plant and environmental conditions (water stress, light, soil nutrient) has generally been examined via reductionist approaches (Etalo et al. 2018). Investigating the influence of changes in soil microbiota on plant performance, especially metabolite accumulation, could provide new frames of reference for microbial engineering and agricultural applications. Although the potential impacts of microbial inoculants on plant metabolites and their synthetic pathways have been widely explored (Karthikeyan et al. 2009, del Rosario Cappellari et al. 2013, Latef et al. 2020), limited information is available concerning the identities and roles of resident microbial taxa that might be influenced by microbial inoculation to contribute indirectly to plant secondary metabolite accumulation. The model-based approach adopted in the present study revealed key taxa that might lead to improved flavonoid and triterpenoid accumulation if increased (mostly members of the phylum *Proteobacteria*) or decreased (such as *Actinobacteriota* and *Chloroflexi*), thus providing a reference for selecting fertilizer regimes. Appropriate fertilization regimes selected by using the microbial indicators in the model were further validated by the observed variation of flavonoid concentrations and yields (Figure 6.2). For example, the OCB1 and OMF2 treatments, which promoted the abundance of potential microbial indicators selected by this model did indeed increase the concentration and yield of flavonoids (Figure 6.2). In contrast, the OMF3 treatment, which was excluded by this model, decreased flavonoid concentration and yield (Figure 6.2, Figure 6.5B). These findings indicate that the prediction of key soil microbiota might be used to identify indicators for devising appropriate soil management strategies. The impacts of the different fertilization regimes on the potential microbial indicators support this conclusion: compared with the treatment with organic fertilizer alone, the top 10 positive microbial indicators were higher at low fertilizer levels but lower at high fertilizer levels in the treatments containing inoculants. Similar patterns of variation were observed for flavonoid levels (Figure 6.2) and the negative potential microbial indicators. Consequently, the identification of potential microbial indicators may be useful not only for screening optimal management strategies, but also for avoiding less beneficial plant-soil interactions.

Pot experiments were performed to compare the robustness of this model-based approach under different conditions. The results indicated that the contributions of some common microbial indicators to metabolite accumulation differed depending on the conditions, whereas other candidates played nearly identical roles in the pot and field experiments. The pot and field experiments had the same soil, plants, and fertilizer but different microbial treatments (Figure S2, the pot experiment contained more inoculant types). Between the two experiments, 20% of the potential microbial indicators had identical effects (positive or negative) on flavonoid accumulation. Consequently, the approach adopted in this study provides a new perspective for understanding the

relationship between soil microbiota and plant metabolites compared with the commonly used linear correlation (Pineda et al. 2020) or principal component analyses (Badri et al. 2013, Chaparro et al. 2013). Because of the compositional nature of soil microbiome data, simple correlation analyses may lead to misleading significant relationships between soil microbiome components and plant performance (Gloor et al. 2017). Future studies employing a series of experiments (i.e. identification of potential microbial indicators, isolation of beneficial microorganisms, successive application in plant and soil system, and comparison of different conditions) are needed to verify the reproducibility, persistence, and resilience of the beneficial impacts derived from soil microbiota. In addition, testing the impact of key microbial indicators on plant metabolism across a range of soil conditions could foster a better understanding of the relationship between belowground communities and aboveground plant traits.

In summary, our findings emphasize that the impacts of microbial inoculation on the growth and the accumulation of metabolites in *C. paliurus* are part of a joint contribution that includes leaf stoichiometric traits and specific changes within the soil microbiota. Furthermore, our model-based approach can identify potential soil-borne microbial indicators for appropriate fertilization regimes and improved plant performance. The identification of microbial indicators may provide a new avenue for selecting appropriate management strategies with the aim to aid plant metabolite production. The model-based approach adopted here can also be further developed and extended toward the examination of other biological interactions, such as system-wide drivers of plant disease incidence, stress responses and crop productivity.

Supplementary material

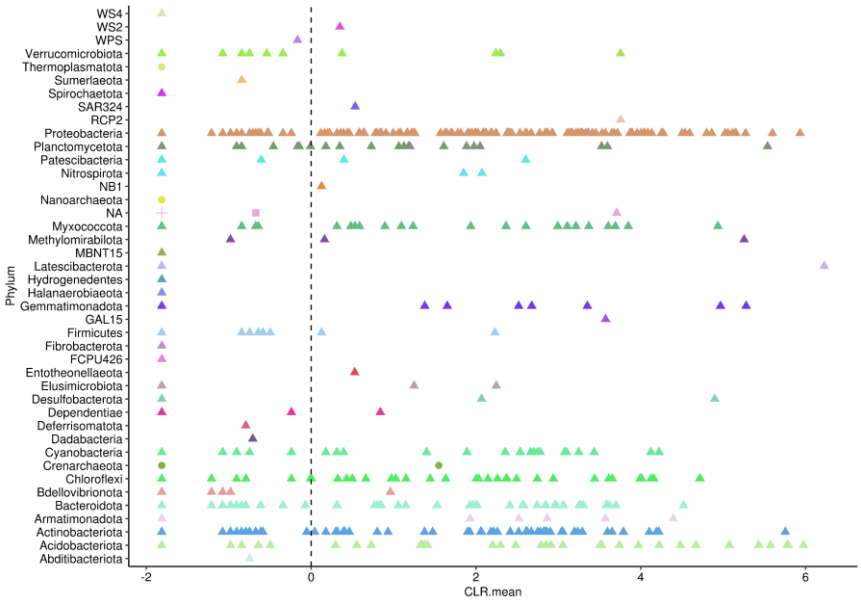


Figure S1 Resident microbiome in soil that not received any fertilizer after three years. CLR.mean: the mean value of microbial abundance after Centered Log-Transformation (CLR). The microbes on the right of the dashed line indicate the increase of their abundances after three years.

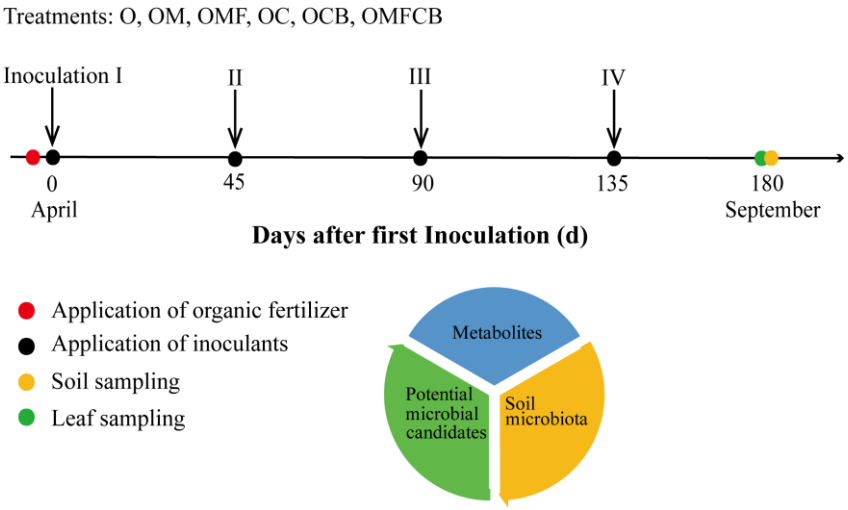


Figure S2 Experimental design of pot experiment. The soils and organic fertilizer for pot experiment were the same as in the field. And both experiments experienced the same climate condition and similar inoculation date (every 45 days) in 2018.

The treatments were: O: single application of organic fertilizer; OM: combined application of organic fertilizer and *Bacillus megaterium*; OC: combined application of organic fertilizer and *Azotobacter chroococcum*; OMF: combined application of organic fertilizer and *B. megaterium* and *Pseudomonas fluorescens*; OCB: combined application of organic fertilizer and *A. chroococcum* and *Azospirillum brasilense*; OMFCB: combined application of organic fertilizer and all four strains.

Sampling: For each treatment, three soil samples (0-10 cm) and three leaf samples were randomly collected from healthy pots, and 16S rRNA sequencing and estimation of metabolites were subsequently conducted.

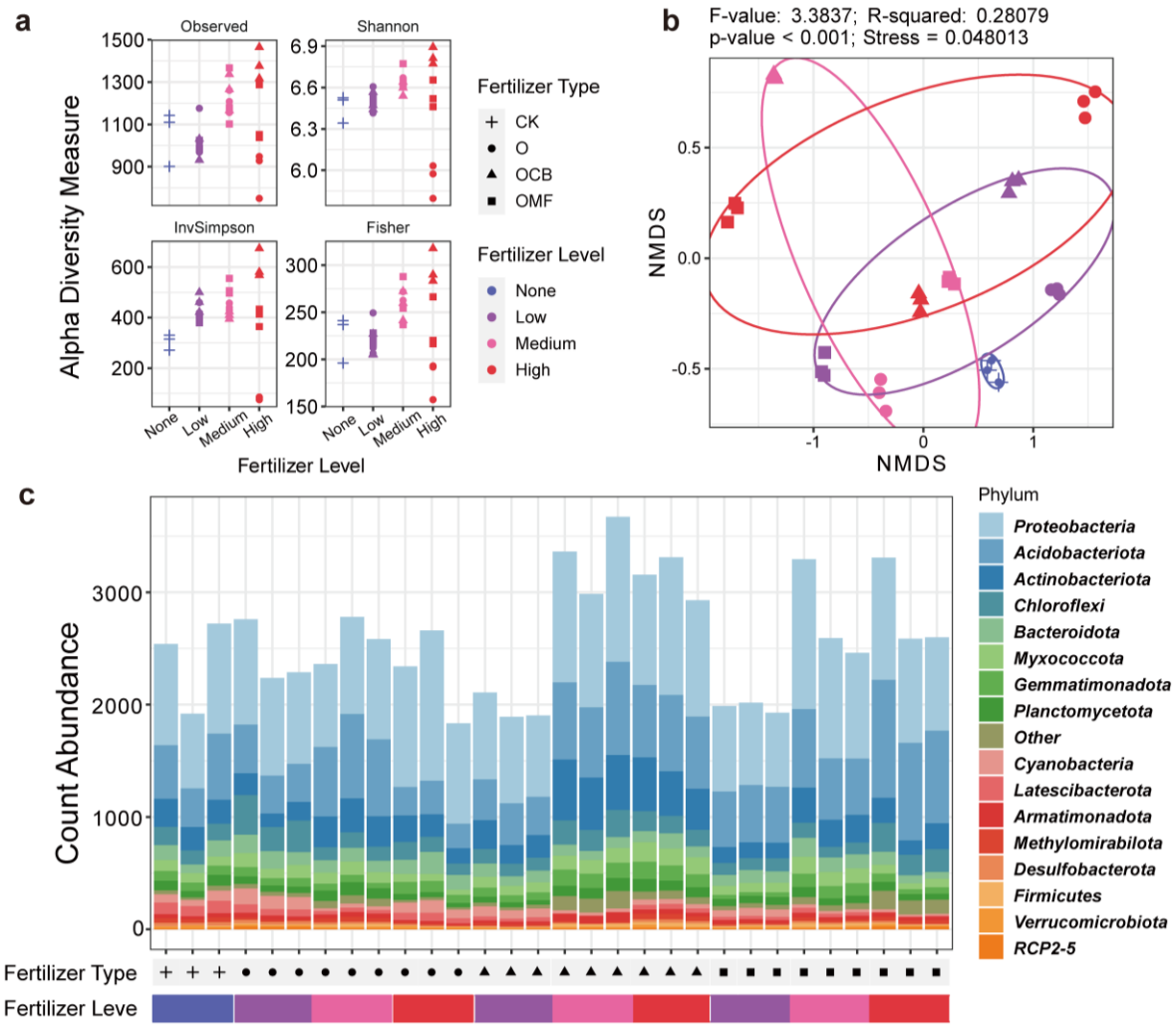


Figure S3 Effects of different fertilization regimes on resident soil bacterial alpha diversity (A), beta diversity (B), and community composition on phylum level (C). Fertilizer type: O = only organic fertilizer, OMF = organic fertilizer and inoculants containing both *Bacillus megaterium* and *Pseudomonas fluorescens*, OCB = organic fertilizer and inoculants containing both *Azotobacter chroococcum* and *Azospirillum brasilense.* Fertilizer level: Low = 10^7 cells + 0.5 kg organic fertilizer per plant, Medium = 10^8 cells + 1.0 kg organic fertilizer per plant, High = 10^9 cells + 1.5 kg organic fertilizer per plant; CK: non-fertilization.

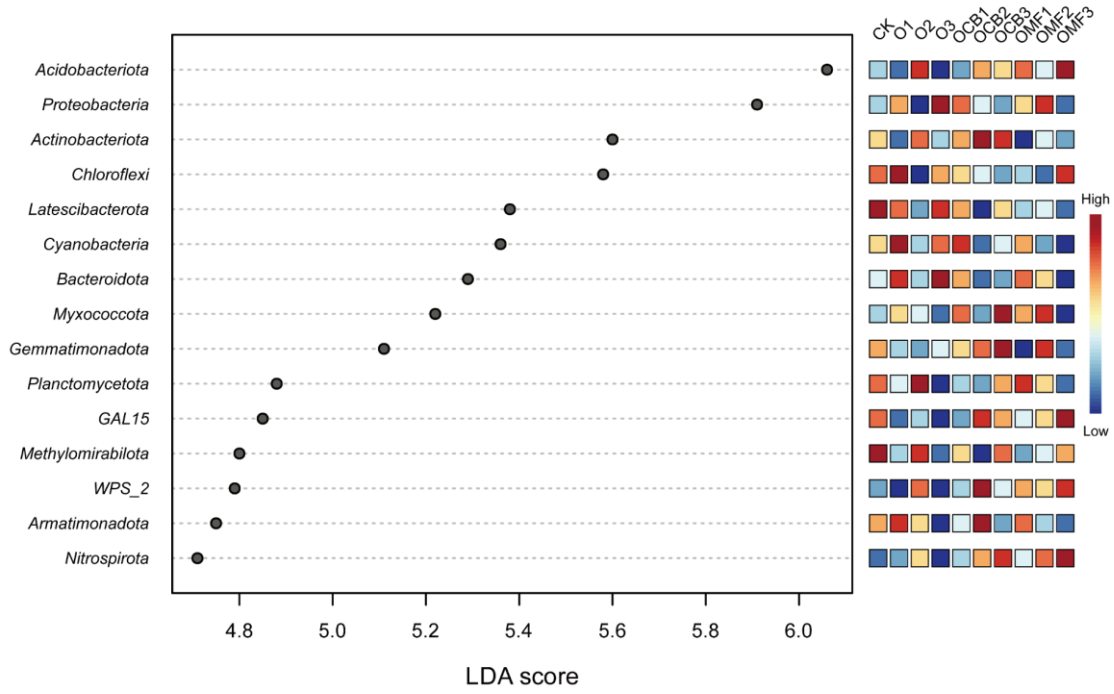


Figure S4 Differentiation of all fertilization regimes in affecting the community abundance on phylum level by linear discriminant analysis (LDA). Fertilizer type: O = only organic fertilizer, OMF = organic fertilizer and inoculants containing both *Bacillus megaterium* and *Pseudomonas fluorescens*, OCB = organic fertilizer and inoculants containing both *Azotobacter chroococcum* and *Azospirillum brasilense*. Fertilizer level: Low = 10^7 cells + 0.5 kg organic fertilizer per plant, Medium = 10^8 cells + 1.0 kg organic fertilizer per plant, High = 10^9 cells + 1.5 kg organic fertilizer per plant. CK: non-fertilization. O1, O2, O3 indicate the organic fertilizer was set at low, medium, and high level, respectively. Same as OCB and OMF.

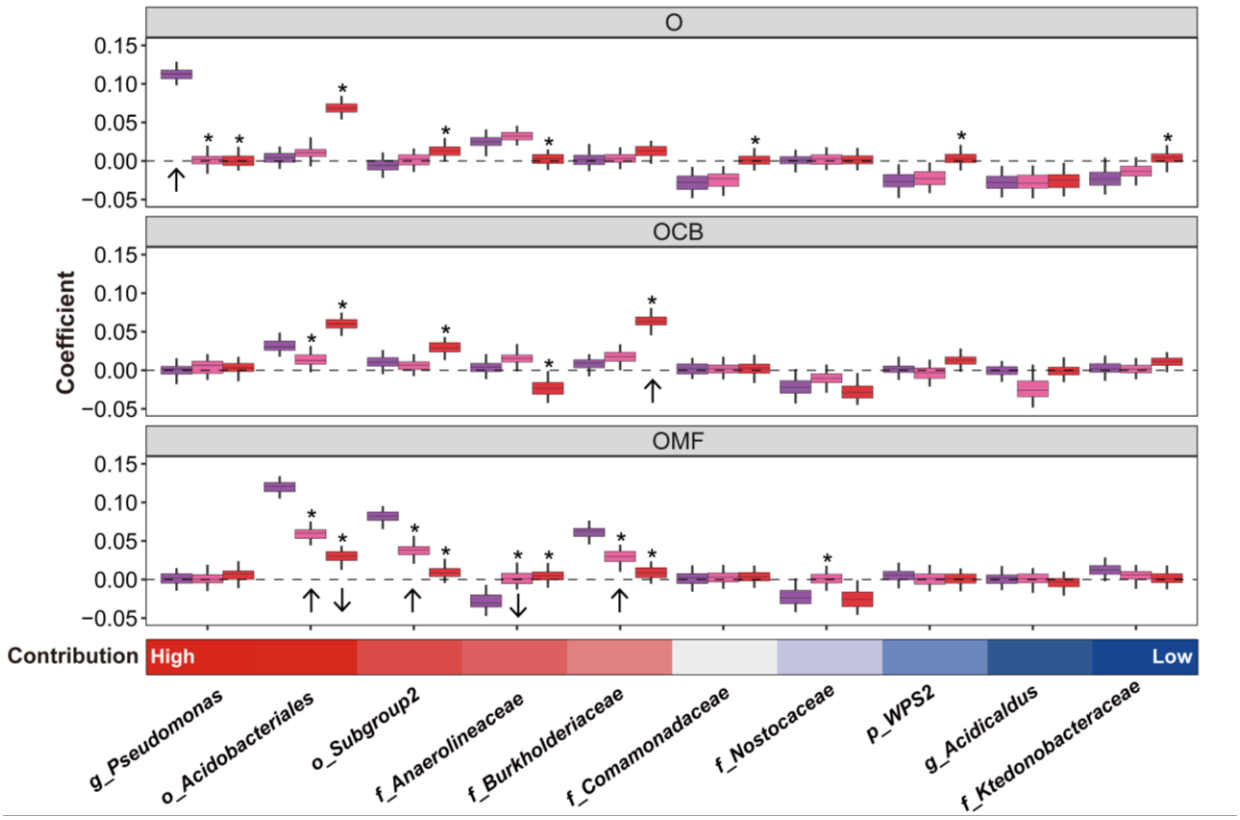


Figure S5 The top 10 bacterial taxonomic contributors affected by different fertilization regimes, * indicates significant difference between fertilizer levels; ↑indicates the fertilizer increased the influence of taxa by compared to other fertilizers at same level; ↓indicates the treatment decreased the influence of taxa by compared to other fertilizers at same level. Fertilizer type: O = only organic fertilizer, OMF = organic fertilizer and inoculants containing both *Bacillus megaterium* and *Pseudomonas fluorescens*, OCB = organic fertilizer and inoculants containing both *Azotobacter chroococcum* and *Azospirillum brasilense.* Fertilizer level: Low = 10^7 cells + 0.5 kg organic fertilizer per plant, Medium = 10^8 cells + 1.0 kg organic fertilizer per plant, High = 10^9 cells + 1.5 kg organic fertilizer per plant.

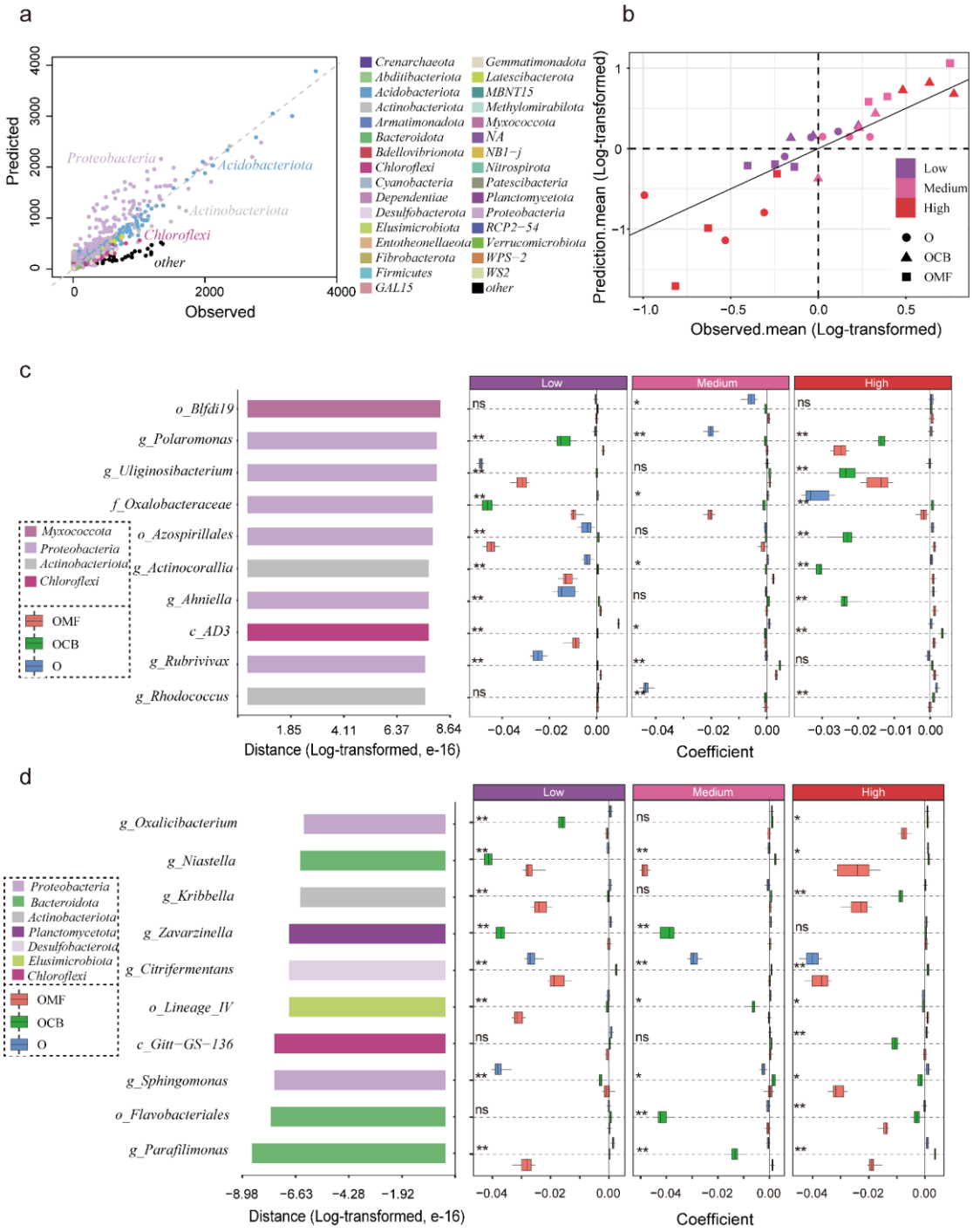


Figure S6 Prediction of optimal triterpenoid accumulation by microbial data assosciated with different fertilization regims in GJAM. (A) Predicted and observed abundance of all phyla in stimulating the accumulation of triterpenoid. To achieve the highest accumulation of triterpenoid in this model, the abundance of the microbes above the dashed line should be increased, while the abundance of the microbes under the dashed line should be decreased. (B) Differenciation of all fertilization regimes in affecting the accumulation of triterpenoid with attribution of microbes. The treatments above the solid line indicated that they resulted a positive effect on the accumulation of triterpenoid. (C) Identification of top 10 taxa that assisted the accumulation of triterpenoid, and how different fertilization regimes affected them. (D) Identification of top 10 taxa that impeded the accumulation of triterpenoid. *: $p < 0.05$; **: $p < 0.01$; ns: no significance.

Table S1 The chemical properties of organic fertilizer used in this study.

Soil properties	pH	Total organic matter	Total N	C/N ratio	P_2O_5	K_2O
Contents	6.68	41.20%	1.70%	24.2	0.8%	1.1%

Table S2 Fertilization regimes in the field experiment.

Fertilizer Type		Fertilizer Level			
		None	Low	Medium	High
O	Inoculum (cell·plant^{-1})	/	/	/	/
	Organic fertilizer (kg·plant^{-1})	/	0.5	1	1.5
OMF	Inoculum (cell·plant^{-1})	/	10^7	10^8	10^9
	Organic fertilizer (kg·plant^{-1})	/	0.5	1	1.5
OCB	Inoculum (cell·plant^{-1})	/	10^7	10^8	10^9
	Organic fertilizer (kg·plant^{-1})	/	0.5	1	1.5

Fertilizer type: O = only organic fertilizer, OMF = organic fertilizer and inoculants containing both *Bacillus megaterium* and *Pseudomonas fluorescens*, OCB = organic fertilizer and inoculants containing both *Azotobacter chroococcum* and *Azospirillum brasilense.*

Table S3 Effects of different fertilization regimes (type and level) on net growth as measured by plant height or stem basal diameter and on nutrient balance in leaves.

Fertilizer type	Fertilizer Level		Plant height (cm)	Ground diameter (mm)	Carbon$^{n.s.}$	Nitrogen (g·kg^{-1})	Phosphorus	C/N	C/P	N/P$^{n.s.}$
O	None	(CK)	115.22 c	26.28 d	474.3	18.43 b	1.44 e	26.0 a	328.8 a	12.7
	Low	(O1)	119.08 c	32.09 bc	479.1	20.26 ab	1.70 bd	23.8 ab	285.1 bd	12
	Medium	(O2)	150.27 b	32.46 bc	472	20.44 ab	1.73 bc	23.3 ab	273.7 cd	11.8
	High	(O3)	135.52 bc	32.13 bc	469	19.04 ab	1.65 bd	24.8 ab	284.2 bd	11.5
OCB	None	(CK)	115.22 c	26.28 d	474.3	18.43 b	1.44 e	26.0 a	328.8 a	12.7
	Low	(OCB1)	142.35 bc	33.87 bc	471.4	19.87 ab	1.59 ce	24.8 ab	297.5 bc	12.5
	Medium	(OCB2)	185.25 a	39.64 a	474.3	22.81 a	1.96 a	20.8 b	242.5 e	11.1
	High	(OCB3)	128.77 bc	31.74 c	470.5	21.7 ab	1.75 b	21.7 ab	269.2 cd	12.5
OMF	None	(CK)	115.22 c	26.28 d	474.3	18.43 b	1.44 e	26.0 a	328.8 a	12.8
	Low	(OMF1)	134.07 bc	34.32 bc	470.5	19.61 ab	1.54 de	24.2 ab	305.5 ab	12.7
	Medium	(OMF2)	174.59 a	37.17 ab	470.3	21.83 a	1.76 b	22.0 ab	268.2 d	12.4
	High	(OMF3)	140.23 bc	32.25 bc	472.3	19.35 ab	1.72 bc	24.4 ab	274.1 cd	11.3

$^{n.s.}$: no significance. Fertilizer type: O = only organic fertilizer, OMF = organic fertilizer and inoculant containing both *Bacillus megaterium* and *Pseudomonas fluorescens*, OCB = organic fertilizer and inoculant containing both *Azotobacter chroococcum* and *Azospirillum brasilense.* Fertilizer level: Low = 10^7 cells + 0.5 kg organic fertilizer per plant, Medium = 10^8 cells + 1.0
kg organic fertilizer per plant, High = 10^9 cells + 1.5 kg organic fertilizer per plant.

Chapter 7 General discussion

Introducing beneficial microbes to the degraded plant-soil system has great potential as an environmentally friendly approach to improve crop yield and quality. However, the effect of soil beneficial microbes (SBM) is highly dependent on the microbial population, species, host plant phenotype, and can be highly variable in different soil types and environmental conditions. This makes it very challenging to develop effective ways of applying SBM to improve degraded soil conditions (Solano et al. 2006, Lugtenberg and Kamilova 2009, Kavamura et al. 2013). In this study, we sought to screen the most appropriate inoculant type and concentrations based on SBM combinations, survivals, and their effects on soil biochemical properties and plant growth performance, especially for improving the plant medicinal value in degraded conditions. Furthermore, we investigated the impacts of microbial inoculants on the resident soil microbial community and the ecological role of the reshaped soil microbiome in regulating plant performance and quality. Finally, we identify the key microbial indicators for selecting appropriate management strategies and improving desired plant products.

Screening the most appropriate inoculants and examining their practical effects in pot and field experiments

To select the appropriate inoculum species, it is vital to know the main categories and traits of SBM. Here, we utilized four commercial SBM strains, M: *Bacillus megaterium*; F: *Pseudomonas fluorescens*; C: *Azotobacter chroococcum*; B: *Azospirillum brasilence* to improve soil quality and *C. paliurus* productivity in China's subtropical area. These strains can be categorized based on their effects on soil nutrients or plant phytochemical responses. For instance, *B. megaterium* and *P. fluorescens* are known as phosphate-solubilizing bacteria (PSB), and *A. chroococcum* and *A. brasilence* are known as N_2-fixing bacteria (NFB) because of their contributions to P and N respectively availability in soil. From the perspective of their roles in regulating plant performance, *P. fluorescens* and *A. brasilence* can be categorized as phytostimulatory strains as they are able to modulate the host plant by changing plant physiology and metabolic profiles. *B. megaterium* and *A. chroococcum* are usually categorized as nutrient-enhancing strains as they can increase plant nutrient acquisition in degraded land conditions. Consequently, the application and combination of these strains should be able to (i) improve soil nutrient characteristics in degraded land; (ii) increase plant productivity and adaptability in degraded land.

Although the application of microbial inoculants has been widely accepted as a means to improve soil restoration and plant growth enhancement, the shortages of available N and P in C-deficient soils make such habitats poorly suited from SBM establishment and propagation. In **Chapter 2**, we investigated the effects of single bacterial additions (SBA) and mixed bacterial additions (MBA) on soil biochemical properties, as well as the ability of inoculated strains to survive in the soils collected from degraded land. Our results indicated that MBA performed

better than SBA in terms of inoculum population dynamics in the soil, although populations did decrease over time due to resource limitation. We speculated that the population growth of MBA could be stimulated by co-inoculation with different strains, and their synergistic effects activated under the circumstances of limited available C and N resources in the microcosms (Wani et al. 2007, Yu et al. 2012). Similar studies have reported that mixed microbial cultures allowed their components to interact with each other synergistically via physical or biochemical activities, thereby improving viability and their efficiency in soil (Vassilev et al. 2001, Shanmugam et al. 2014, Hu et al. 2016). This result can help choose the inoculant type (MBA) and frequency (30–45d) of fertilization when applying bio-fertilizer in such soils. Co-inoculation with PSB and NFB in soil results in more interactions of inoculants, such as the production of enzymes and organic acid, although more resources would be consumed than when these organisms were used alone (Paerl and Pinckney 1996, Yu et al. 2012, Wei et al. 2018a). The results of **Chapter 3** and **4** also confirmed that mixed inoculants performed better than single inoculants in stimulating plant growth and soil nutrient properties. For instance, in **Chapter 4**, plant growth was most stimulated by mixed inoculants MFCB (co-inoculation with four strains), resulting in significantly taller plants with a larger ground stem diameter throughput the whole inoculation period. Dual inoculants such as MF (*B. megaterium* and *P. fluorescens*) also showed significant advantages over single inoculant M in improving soil enzyme activities at certain time points.

Selecting the appropriate microbial inoculants is an essential step toward achieving goals related to plant production and soil quality improvement in degraded land. For instance, as a multi-functional woody plant, *C. paliurus* could be utilized for timber use, tea material, as well as a natural pharmacy resource (Fu et al. 2015). In **Chapter 3**, we found that the inoculant type influenced plant growth promotion, biomass allocation, and the accumulation of bioactive compounds. For timber use, a fertilization strategy in favor of vegetative growth, reflected in tree height, diameter, and stem biomass, should be considered as a priority. As shown in our work, MFCB and MF treatments improved growth and above-ground biomass accumulation of *C. paliurus*, making them good candidates in *C. paliurus* plantations used for timber production. For medicinal plants, it is vital to obtain a high yield of bioactive compounds. However, only limited information is available concerning the impact of inoculant type on the concentrations of medicinal components (Dadrasan et al. 2015, Deng et al. 2019b). In the present study, inocula related to NFB (i.e. C, CB: *Azotobacter chroococcum* and *Azospirillum brasilense*, MFCB) resulted in higher yields of bioactive compound than that in PSB and the control, and the highest production of leaves was noted in MFCB, which yielded twice as much as the control. In addition, the biomass improvement of *C. paliurus* leaves, which are the main organs for the production of medicinal components, can also lead to a high

yield of metabolites per plant.

With regard to inoculation frequency, we hypothesized that the beneficial effects of microbial inoculants on soil nutrients and plant growth would increase with repeated applications. In **Chapter 4**, we found that periodic inoculations mostly increased soil available nutrients during the first 10–90 days. Although the treatments MFCB and CB appeared to be most pronounced at the last two sampling times, the benefits of inoculation generally decreased over time. This indicates the effects of microbial inoculants on soil functioning were transient. Given the resilience and resistance of the resident microbiome (Griffiths and Philippot 2013), we speculate that this decrease could be due to changes in the soil microbial community. PCoA and community type analyses confirmed that the resident bacterial community in the bulk soil underwent shifts over the course of the first 90 days but showed no significant dissimilarities at the last two sampling times. This is in accordance with the observed variation of nitrogenase activity and soil inorganic nitrogen content: no differences were found between inoculation and non-inoculation in the last 90 days. Consequently, the necessity of repeated inoculations should be reconsidered, and while the different microbial inoculants showed distinct impacts on resident microbiome succession, communities ultimately exhibited functional resilience.

Even though introduced microbial inoculants sometimes cannot compete efficiently with native microorganisms in the soil, they can still stimulate root growth and modify plant metabolism at very early stages, thereby potentially generating lasting effects on the root system and associated microbial communities (Bashan 1999). In **Chapters 4** and **5**, microbial inoculations significantly promoted *C. paliurus* growth and reshaped root morphological traits compared to non-inoculated seedlings after the inoculation period in Baima (Wang et al. 2021b). However, the growth-promoting effect was highly variable across time and inoculums and not maintained when the seedlings were transplanted to Taizhou. Thus, it may be necessary to adopt strategies of multiple microbial inoculations when transplanting and establishing a plantation at a different site.

Periodic inoculations impact the resident microbial community

Soil beneficial microorganisms interact intimately with the roots of the host plant and affect the ecological adaptability of the plant to its environment. Nonetheless, these beneficial effects can be weakened by intensive land usage, thereby decreasing the plant's capacity to deal with biotic and abiotic stresses (Strigul and Kravchenko 2006). To maintain the beneficial effects of microbial inoculants on plants and soil, repeated inoculation represents a promising option. However, the native soil microbial community is sensitive to exogenous disturbances

(Hartmann et al. 2015, Suleiman et al. 2016), and invading microbes, such as beneficial microbial inoculants, can alter microbial community succession, composition, function, and diversity (Xiong et al. 2017, Lourenco et al. 2018). Although some studies have examined the impacts of one-off inoculation on the native microbiome, it remains unclear how long and to what extent the periodic inoculations may affect the succession of the resident microbiome in soil.

In **Chapter 4**, we tracked the succession traits of resident microbiome in the bulk soil across the growing season and identified the taxa clusters that responded differently to periodic inoculations of PSB and NFB alone or in combination. Furthermore, we examined the dynamic responses of plant growth and soil functions. Our results showed that the changes in soil nutrients were consistent with the shifts of the resident microbial community, and 57% of the significant variation among treatments occurred during the first 45 days after inoculation. The resident soil bacterial communities appear to exhibit a high level of resilience, but not resistance, to the microbial disturbance caused by periodic inoculations. The initial inoculation disturbed the stability of the resident microbiome, which was as a result more susceptible to subsequent inoculation disturbances. This is in line with discussion presented above that the effects of such amendments on below-and aboveground are transient. Surprisingly, in the present study, the second inoculation still left a footprint on the resident community, resulting in an increase in the number of effected cluster types in the inoculated soils compared with the control. This finding also confirms a previously proposed hypothesis that the second disturbance by the same invader could persist longer or even assimilate into the community (Mallon et al. 2018).

Different inoculant types (single/mixed) exhibited disparate effects on plant and soil characteristics, which could be linked to, or lead to, different changes of the resident community. In **Chapter 4**, we found that different inoculants (single/mixed) transiently modified the resident community during the first 45 days after inoculation. Co-inoculation could leave a different footprint on the resident microbiome compared to single inoculation, because mixed inoculants may occupy different ecological niches in the resident community comparing to single application of each strain (Paerl and Pinckney 1996, Yu et al. 2012, Wei et al. 2018a). The nature of such differences could also be due to the feedback of changed soil environments and plant performance. Soil pH and the C/N ratio were the main factors underlying this impact, followed by nitrate. Supporting these results, soil C/N at 30 days after the first inoculation was higher for single inoculants compared to for mixed inoculants. Differences in pH between the single- and mixed-inoculant treatments were not significant. It should also be recognized that other environmental factors that were not assessed in this study could be driving these differences. Interestingly, the changes to the resident communities after application of single and mixed inoculants were only observed for a

short period of time. Eventually, the resident communities generally exhibited similar traits regarding to the community structure and function. In summary, we found that inoculation significantly impacted microbial community succession as compare to non-inoculated controls, resulting in different cluster types and composition shifts, thus providing a new insight the interactions between resident microbes and intruders. The changes in the resident community mostly reflected the initial disturbance of inoculant addition and partially explained subsequent variation in soil nutrients and plant growth.

In the review of previous studies, we found that the impacts of periodic microbial inoculation on the indigenous community were highly dependent on environmental factors, microbial diversity, and soil type (Schreiter et al. 2014, Xun et al. 2015). For instance, Schreiter et al. (2014) applied a combination of analysis methods (PCR-DGGE and 16S rRNA gene pyrosequencing) to evaluate the impacts of periodically introduced *Pseudomonas* sp. on the resident community and found that soil type played a more important role than the inoculant itself in determining the impact of inoculation on the resident community. Furthermore, the long-term impact of microbial inoculation on the resident community determinates the subsequent performance of the plant and final productivity. Zhang et al. (2019) found that pre-colonization of PGPR (*Bacillus velezensis* NJAU-Z9) in potting soil can lead to the changes of resident bacterial and fungal community after transplanting pepper pots into the field, and such changes were associated with enhanced plant yield. The introduced PGPR not only survived in the field, but also induced higher relative abundance of beneficial genera in the resident community associated with improved crop yield.

In total, microbial inoculants can impact the resident community that can exhibit a varying degree of resistance and resilience, and the outcome is highly dependent on soil type, inoculant type, and native community diversity.

Linking the reshaped microbiome to soil functioning and plant performance

Introducing soil beneficial microbial inoculants can improve soil fertility and leave a footprint on the resident soil microbiome. In turn, the changes in composition and diversity of the soil microbial community can impact soil biogeochemical processes, especially for soil enzyme activities and N processes (Zheng et al. 2019). For instance, the alteration of microbial community composition and the populations of specific microbes, such as ammonia-oxidizers (Li et al. 2018), N-fixers, and P-solubilizers (Bargaz et al. 2018) determines a number of crucial soil functions (Don et al. 2017). On the one hand, the introduced microbes could outcompete specific taxa that share similar ecological niches and compete for similar resources in the soil (Mallon et al. 2018, Mawarda et al. 2020).

On the other hand, SBM in bio-fertilizer can induce positive effects on beneficial microbial groups such as *Pseudomonas* spp. and *Bacillus* spp., thereby impacting for instance soil nutrient status (Xiong et al. 2017, Wang et al. 2021a).

In **Chapter 4**, the relative abundances of families like *Xanthomonadaceae* significantly increased in the treatments with PSB, suggesting that the introduction of PSB facilitated these specific resident populations, as was also observed in a previous study (Kuramae et al. 2011). We also found that pH was one of the dominant factors explaining the succession of the resident community over time. Supporting this result, soil pH in inoculated treatments significantly differed from that in non-inoculated soil after the first and the fourth inoculations. This might be the outcome of the inoculation with PSB. The PSB possess the ability of producing organic acids during the decomposition of soil organic matters, which is associated with the release of P from mineral-bound complexes such as $AlPO_4$ and $FePO_4$, thus leading to a decrease of soil pH and a change the related nutrient contents (Orhan et al. 2006). Furthermore, we identified taxonomic markers for each cluster in the inoculated soils, with the phyla *Proteobacteria* and *Bacteroidetes* generally having copiotrophic strategies with rapid growth responses to resource availability (Fierer et al. 2007). In the results of **Chapter 4**, these phyla were enhanced during the first 45 days after inoculation, which is also the period that showed rapid changes in soil nutrients.

It is well accepted that beneficial microbial inoculants can interact intimately with the roots of the host plant and affect the ecological adaptability of the plant to its environment. In fact, microbial inoculation can be seen as an anthropogenic version of the plant's natural "call for help" mechanisms in degraded land. Plant growth-promoting effects are usually attributed to enhanced plant nutrient acquisition (Wang et al. 2019c), reshaped root morphology (Wang et al. 2021b), increased photosynthetic rate and chlorophyll content (Mishra et al. 2020), and indole acetic acid production (Bhardwaj et al. 2014, Kuramae et al. 2020). However, less is known about the role of the reshaped soil microbiota in regulating plant performance. The results in **Chapter 4** indicated that periodic inoculation did not increase the benefits of microbial inoculants on plant growth as we expected, which is in accordance with the succession traits of soil microbiome: the initial inoculation played a more important role in influencing the whole system, and the native microbial community exhibited traits of resilience to subsequent inoculations. Hence, the impacts of microbial inoculants on the resident community could lead to the subsequent changes in not only soil functioning but also plant performance.

Implications for improving plant medicinal value

For medicinal plant cultivation, it is important to optimize management strategies such as to achieve reliably high yields of bioactive compounds. However, the accumulation of bioactive compounds, especially for secondary metabolites, is highly dependent on the complex interactions with abiotic and biotic environment (Verhagen et al. 2004, Vorholt 2012). Here, we co-inoculated phytostimulatory strains (*Azospirillum* sp., *Pseudomonas* sp.) and nutrient-enhancing strains (*Bacillus* sp*., Azotobacter* sp.) to improve the biochemical properties of the soil and increase plant metabolic production. Our results in **Chapters 3**, **5**, and **6** show that the yield of flavonoids and triterpenoids is associated with soil nutrient availability, soil microbiota, plant root morphology, plant nutrient stoichiometry, leaf biomass, and biofertilizer type and level. It is therefore important to integrate all the factors when trying to optimize the production of bioactive compound in *C. paliurus*.

Firstly, our attention should be paid on the trade-off between plant primary production, accumulation of plant secondary metabolites, and improving degraded land conditions. Plants grown in degraded land frequently suffer from abiotic and biotic stresses that constrain their primary growth, but may stimulate the accumulation of plant secondary metabolites involved in the plant's stress response. Thus, despite the problems associated with degraded land, such soils may hold potential for the production of medicinal plants with high levels of desired plant secondary metabolites. However, degraded soil conditions may affect the plant's growth capacity to an extent that yields become too low, and extreme stress may inhibit leaf biomass accumulation, which is critical given the fact that target metabolites are produced in and harvested from leaf tissues. The goal of SBM application in degraded lands should be to achieve higher concentration of target metabolites in the medicinal plants without compromising primary growth. SBM application could for instance lead to higher plant primary productivity but a lower accumulation of secondary metabolites. In other words, if the amendment strategies enrich the soil environment, the plants probably accumulate less concentrated in desired metabolites than under the unamended degraded conditions.

Our results of the pot experiment (**Chapter 3**) showed that the yield improvement of metabolites can be attributed to increased leaf biomass as opposed to a higher concentration of the metabolites in the leaves. The accumulation of leaf biomass was also correlated with plant nutrient acquisition. The results of the field experiments (**Chapter 5** and **6**) showed that the inoculation with SBM and organic fertilizer at low (10^7 cells per plant) and medium (10^8 cells per plant) levels can improve both the concentration and yield of bioactive compounds in *C. paliurus* leaves. Crucially, the output of bioactive compounds was associated with leaf nutrient stoichiometry: medium values of

the C/N (approximately 24) and C/P (approximately 280) ratios were linked to the highest production of flavonoids and triterpenoids. Hence, to optimize the production of metabolites in *C. paliurus* leaves, introducing the beneficial microbial inoculants should be able to manipulate the plant nutrient stoichiometry to an appropriate level and improve the leaf biomass without compromising the accumulation of secondary metabolites.

Secondly, it is well-known that the soil microbiome impacts plant performance in many aspects. However, we have far less knowledge related to how we can steer the soil microbiome to modify plant metabolites by introducing probiotic consortia and how to identify microbial indicators for aboveground changes. Crucially, investigating the influence of changes in soil microbiota on plant performance, especially metabolite accumulation, provides new frames of reference for microbial engineering and agricultural applications. Our findings in **Chapter 6** highlight the impacts of microbial inoculation on the growth and the accumulation of metabolites in *C. paliurus* as part of a joint contribution that includes leaf stoichiometric traits and specific changes within the soil microbiota. For instance, when we applied a model-based approach to identify the key taxa that might lead to improved flavonoid and triterpenoid accumulation if they were increased (mostly members of the phylum *Proteobacteria*) or decreased (such as *Actinobacteriota* and *Chloroflexi*), thereby providing a reference for selecting fertilizer regimes. More importantly, the appropriate fertilization regimes selected by using the microbial indicators in the model were further validated by the observed variation of flavonoid concentrations and yields. Thus, the identification of microbial indicators may provide a new avenue for selecting appropriate management strategies with the aim of improving plant metabolite production. From the perspective of practical application, the role of soil microbiome engineering is as important as the introduced beneficial inoculants for stimulating the yield of desired products in the plant.

Conclusions and future prospects

Introducing SBMs to degraded land is a promising strategy for improving plant productivity and soil abiotic and biotic conditions, but there is still a large knowledge gap concerning how to best optimize inoculant efficiency and the interactions with soil resident community and host plants. To bridge this gap, firstly, we conducted a series of soil incubation experiments in the lab to evaluate the population dynamics of four PGPR strains in a C-deficient soil, and we examined their effects at different organic fertilizer levels. Soil C content is a key factor that limits microbial growth. Hence, organic amendments can help introduced microbes to propagate and function in the soil (Shahzad et al. 2014). Exploring the appropriate organic input level for microbial inoculants is crucial to improve

the effects on plant and soil characteristics. In this study, we showed that soil C deficiency limited the growth of PGPR (**Chapter 2**) and organic fertilization increased the effects of PGPR on soil nutrient characteristics (**Chapter 5**). However, fertilizer level could result in different effects under controlled and field conditions: inoculation increased the soil nutrient availability with increasing fertilization level in the lab, but the medium fertilizer level provided optimal growth in the field. Generalized joint attribute model (GJAM) analysis showed that high fertilizer levels reduced the influence of the probiotic consortia on the whole system, with fewer differences observed between fertilizer types. However, it must be mentioned that organic inputs could help PGPR establish stable populations in the poor soil and alleviate environmental stress, but a specific stressed condition might improve PGPR efficiency and certain enzyme activity in the soil. More systematic and dedicated studies will be required to disentangle the nature of the trade-offs involved with organic input and PGPR efficiency in order to optimize amendment strategies for desired production schemes.

Moreover, we investigated the succession of soil-resident microbial community in response to periodic inoculations of PGPR. To date, most studies have focused on the ultimate effects on the native microbial community, but relatively few studies have investigated the succession of the resident community in response to periodic inoculations of PGPR. This kind of bias limits the evaluation of practical application of inoculants in degraded conditions, with the underlying factors driving community succession remaining unclear. Our research in **Chapter 4** showed that initial inoculation plays a more important role in influencing the whole system, and the native microbial community exhibits traits of resilience, but no resistance, to subsequent inoculations. Repeated inoculations did not generally further improve the benefits from microbial inoculants, and the beneficial effects on plant growth were not maintained after transplanting to a different site. Consequently, the effects of repeated microbial inoculations should be further tested as their effectiveness may be highly context-dependent.

Finally, understanding the overall relationship between aboveground and belowground traits can help researchers and farmers make more balanced amendment strategies. Most previous studies have simply considered the linear relationship between a few variables and ignored other important factors and underlying mechanisms. For instance, many studies have attributed the enhanced plant growth or soil nutrient to the microbial inoculant itself, while the role of other parameters such as the reshaped soil microbiome are often overlooked. Consequently, a model-based approach should provide a more general perspective for selecting the key determinants of improved ecological indexes and understanding the above-belowground interaction. Here, we applied a model-based approach to link plant probiotics, soil microbiome properties and plant production (**Chapter 6**). We found that specific soil microbial taxa can be identified as potential indicators of appropriate fertilization-inoculum

combinations for optimal plant metabolite production, indicating that probiotic consortia can modulate plant metabolites by conditioning the soil microbiome. This provides a new perspective toward understanding above-belowground interactions. Our research should therefore help researchers understand the role of soil microbes in eliciting specific desired plant products and help farmers design and apply the most appropriate management strategies.

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Summary

Land degradation usually leads to a reduction in soil fertility, decline of plant productivity, and loss of biodiversity. Conventional management practices, such as the application of inorganic fertilizer, have been linked with negative environmental impacts. Introducing beneficial microbial inoculants to degraded lands represents a promising strategy, but their effects on plant-soil system performance are highly context-dependent and influenced by many factors, such as the characteristics of the resident microbial community. The work described in this thesis focused on degraded land with shortages of organic matter and nutrients. We combined the use of microbial inoculants with organic fertilizer to investigate the effects of inoculant type, inoculation period, and organic fertilizer level on plant-soil performance and the ecological role of the resident soil microbial community.

In **Chapter 2**, we examined the individual and combined effects of four commercial strains (M: *Bacillus megaterium*; F: *Pseudomonas fluorescens*; C: *Azotobacter chroococcum*; B: *Azospirillum brasilence*) on soil nutrient properties and their survival dynamics in C-deficient soils collected from degraded land. Results showed that mixed microbial consortia performed bettered than single strains in terms of inoculum population dynamics in the soil, although introduced populations still decreased after 45 days due to resource limitation. This indicates that mixed microbial cultures may allow their components to interact with each other synergistically via physical or biochemical activities, thereby improving viability and their efficiency in soil, but the effects are limited by resource availability over time.

To subsequently test their synergistic effects, **Chapters 3** and **4** assessed the practical applications of the selected inoculants using a pot experiment. The results of **Chapters 3** and **4** confirmed that mixed inoculants performed better than single inoculants in stimulating plant growth and soil nutrient properties. However, in **Chapter 4,** the effects were only observed during the first 90 days and did not increase with repeated inoculations. In addition, the single and mixed inoculants transiently modulated the structure of the resident microbial community, which showed resilience to subsequent inoculations. These results suggest that, although the effects are different between mixed and single inoculants, their practical effects on plant-soil performance are time-limited and influenced by the resident community traits. The initial inoculation plays a more important role in influencing the whole system, and repeated inoculations did not generally further improve the benefits from microbial inoculants.

Chapters 5 and **6** examined the practical effects of mixed microbial inoculants under field conditions after one year and three years, respectively. A different pattern was found between field and control experiments: inoculations increased the soil nutrient availability with increasing fertilizer level in the soil incubation test, but the medium

fertilizer level provided the most optimal plant growth in the field, which was associated with plant nutrient acquisition and root morphology. This suggests that the effects of microbial inoculants can be influenced by other factors under field conditions, such as the activities of resident microbes. **Chapter 6** further used a modeling approach to identify specific soil microbial taxa that can act as potential indicators relating to the relative success of microbial inoculant application. These indicators represent taxa that may assist or impede the ability of inoculants to improve plant metabolite levels.

In summary, as an alternative option to conventional strategies, microbial inoculant applications show great potential for improving degraded land productivity, but the effects of beneficial microbial inoculants are highly context-dependent. The inoculant type, concentration, inoculation frequency, and soil-resident microbes all can play important roles in affecting the ultimate effects of microbial inoculants on both soil nutrient properties and plant productivity. Integrated analysis of those key factors can help determine the most appropriate management strategies for improving such degraded ecosystems.

Samenvatting (Dutch summary)

Bodemdegradatie leidt meestal tot een afname in bodemvruchtbaarheid, een vermindering in plant productiviteit en een verlies van biodiversiteit. Gebruikelijke management praktijken, zoals de applicatie van anorganische meststoffen, kunnen negatieve effecten hebben op het milieu. De introductie van goedaardige microbiële inoculaties in gedegradeerde bodems is een veelbelovende strategie voor bodem restoratie, maar de bijkomende effecten op de prestatie van het plant-bodem systeem zijn zeer context-afhankelijk en worden beïnvloed door een verscheidenheid aan factoren, zoals bijvoorbeeld de samenstelling van de microbiële gemeenschap reeds aanwezig in de bodem. Het onderzoek beschreven in dit proefschrift richt zich op gedegradeerde bodems met een tekort aan organische stoffen en nutriënten. Wij hebben het gebruik van microbiële inoculaties en organische meststoffen gecombineerd om het effect van inoculatie type, inoculatie periode en organisch meststof niveau te onderzoeken in relatie tot plant-bodem prestaties en de ecologische rol van de initiële microbiële bodem gemeenschap.

In **Hoofdstuk 2** onderzoeken we de individuele en gecombineerde effecten van vier commerciële stammen (M: *Bacillus megaterium*; F: *Pseudomonas fluorescens*; C: *Azotobacter chroococcum*; B: *Azospirillum brasilence*) op de eigenschappen van bodemnutriënten en hun bijbehorende overlevings-dynamiek in kalium-arme bodems, die verzameld zijn van gedegradeerde bodems. De resultaten laten zien dat gemixte microbiële inoculaties beter presteren dan individuele stammen op zichzelf wat betreft populatiedynamiek in de bodem, al nemen alles populaties af na 45 dagen door een tekort aan nutriënten. Dit laat zien synergistische interacties, via fysieke of biochemische activiteiten, kunnen optreden in microbiële consortia, waarbij de levensvatbaarheid en hun efficiëntie in de bodem wordt verbeterd. Uiteindelijk zijn de effecten gelimiteerd in de tijd door beperkte nutriënt beschikbaarheid.

Om vervolgens de synergistische effecten te testen, bespreken **Hoofdstukken 3** en **4** de praktische applicatie van de geselecteerde inoculaties in een pot experiment. De resultaten van **Hoofdstukken 3** en **4** bevestigen dat gemixte inoculaties beter presteren in het stimuleren van plant groei en bodem nutriënt eigenschappen, dan individuele stammen. Daarentegen werden de effecten in **Hoofdstuk 4** alleen gezien gedurende de eerste 90 dagen en namen deze ook niet toe na herhaaldelijk inoculaties. Bovendien, hadden de individuele en gemixte inoculaties slechts een tijdelijk effect aan de samenstelling van de initiële gemeenschap in de bodem, en bodemgemeenschappen waren weerbaar tegen daaropvolgende inoculaties. Dit suggereert dat, ook al zijn de effecten verschillend tussen individuele en gemixte inoculaties, hun praktische effecten op land-bodem prestatie zijn van tijdelijke duur en

worden beïnvloed door de eigenschappen van de initiële gemeenschap in de bodem. De initiële inoculatie speelt een belangrijkere rol in het beïnvloeden van het gehele systeem en herhaaldelijke microbiële inoculaties hebben over het algemeen geen verdere verbetering gebracht.

Hoofdstukken 5 en **6** bekeken de praktische effecten van gemengde microbiële inoculaties onder veld condities na één en drie jaar, respectievelijk. Wij hebben verschillende patronen gevonden voor veld *versus* controle experimenten: inoculaties verhoogden de nutriënt beschikbaarheid van de bodem in een incubatie experiment met toenemende meststof niveaus, maar het medium meststof niveau zorgde voor een optimale plant groei in het veld, wat geassocieerd was met plant nutriënt opname en wortel morfologie. Dit suggereert dat de effecten van microbiële inoculaties worden door andere factoren beïnvloed onder veldcondities, zoals de activiteit van de initiële bodemgemeenschap. **Hoofdstuk 6** gebruikt een modulering aanpak om dieper in te gaan op het identificeren van specifieke bodem microbiële taxa als potentiële indicatoren gerelateerd aan het gebruik van microbiële inoculaties. Deze indicatoren kunnen een stimulerend of belemmerend effect hebben op het gebruik van inoculaties voor het verbeteren van plant metabolieten.

Samenvattend, microbiële inoculaties zijn veelbelovend alternatieve opties ten opzichte van conventionele strategieën, voor het verhogen van productiviteit en het tegengaan van bodemdegradatie, maar de effecten van goedaardige microbiële inoculaties zijn zeer context-afhankelijk. Het inoculatie type, de concentratie, de inoculatie frequentie en de initiële bodem-gemeenschap kunnen allemaal een belangrijke rol spelen in het beïnvloeden de uiteindelijke effecten van microbiële inoculaties op zowel de bodem nutriënt eigenschappen als plant productiviteit. Geïntegreerde analyses van deze factoren kunnen helpen bij het bepalen van de best passende managementstrategie in aangetaste ecosystemen.

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Publication list

Wang Z, Chen Z, Kowalchuk GA, Xu Z, Fu X, Kuramae EE. 2021. Succession of the resident soil microbial community in response to periodic inoculations. Applied and Environmental Microbiology 87:e00046-21.

Wang Z, Xu Z, Chen Z, Kowalchuk GA, Fu X, Kuramae EE. 2021. Microbial inoculants modulate growth traits, nutrients acquisition and bioactive compounds accumulation of *Cyclocarya paliurus* (Batal.) Iljinskaja under degraded field condition. Forest Ecology and Management 482:118897.

Wang Z, Chen Z, Xu Z, Fu X. 2019. Effects of phosphate-solubilizing bacteria and N_2-fixing bacteria on nutrient uptake, plant growth, and bioactive compound accumulation in *Cyclocarya paliurus* (Batal.) Iljinskaja. Forests 10:772.

Wang Z, Chen Z, Fu X. 2019. Integrated effects of co-inoculation with phosphate-solubilizing bacteria and N_2-fixing bacteria on microbial population and soil amendment under C deficiency. International Journal of Environmental Research and Public Health 16:2442.

Wang Z, Xu Z, Chen Z, Fu X. 2020. Synergistic effects of organic fertilizer coupled with phosphate-solubilizing and nitrogen-fixing bacteria on nutrient characteristics of yellow-brown soil under carbon deficiency. The Journal of Applied Ecology 31:3413-3423. (in Chinese)

Wang Z, Chen Z, Leite Marcio FA, Xu Z, Lin Q, Kowalchuk GA, Fu X, Kuramae EE. Identifying soil microbial indicators relating the use of probiotic microbes to improved plant performance via a model-based approach. (submitted)

Wang Z, Kowalchuk GA, Xiangxiang Fu, Kuramae EE. Roles of microbial inoculants and the associated soil microbiome in restoring degraded land. (submitted)

Curriculum Vitae

Zhikang Wang, born on 15 March 1993 in Anqing, Anhui, China. He grew up in a small village with mountain forests and rivers. Since he was a kid, he showed great interest in plants, animals, and nature view, which motivate him to choose forestry as his major at Nanjing Forestry University. After his bachelor study, he joined the research group of Prof. Xiangxiang Fu and Prof. Shengzuo Fang. In his master thesis, he investigated the effects of bio-fertilizer on plant metabolites and soil nutrient properties, where the plant-soil-microbes interactions attracted his great interest. In 2017, he started his PhD project, his research focused on the impacts of microbial inoculants on the restoration of degraded land quality and plantation productivity. After three years of field and lab work, in 2020, he obtained the scholarship from China Scholarship Council to continue his PhD thesis with the promoters Prof. Eiko E. Kuramae and Prof. George A. Kowalchuk at NIOO-KNAW and at Utrecht University. Now he has finished his PhD thesis and is ready to embrace the science of microbial ecology at the forest scale.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- Responses of resident soil microbiome to continuous PGPR inoculation

Writing of project proposal (4.5 ECTS)

- Effects of PGPR inoculation on soil nutrients, microbial community and *Cyclocarya paliurus* growth in C-deficient soil of southern China

Post-graduate courses (8.3 ECTS)

- Silviculture; Shengzuo Fang, College of Forestry (2017)
- Forest soil microbiology; Hui Sun, College of Forestry (2017)
- Forest soil science; Huanchao Zhang, College of Forestry (2017)
- Experimental design and statistical analysis; Yannan Xu, College of Biology (2017)
- Meta-analysis; PE&RC (2020)
- Introduction to data science with R and R studio; online; PE&RC (2020)

Deficiency, refresh, brush-up courses (3 ECTS)

- Introduction to forestry specialty; Shengzuo Fang, China (2017)
- Program management and career planning; Hua Zhang, China (2017)
- Microbiology; Ben Fan, College of Biology, China (2017)

Laboratory training and working visits (6 ECTS)

- Essay of soil fundamental properties; Nanjing Forestry University (2017)
- Microbial ecology; Institute of soil science, Chinese Academy of Sciences (2018)

Invited review of journal manuscripts (2 ECTS)

- Soil Biology and Biochemistry: major roles of soil biota linked to soil structure and C (2021)
- Science of the Total Environment: role of rare bacteria in soil (2021)

Competence strengthening / skills courses (4.5 ECTS)

- Academic English reading and writing; College of Foreign Language (2017)
- Oral English and presentation; College of Foreign Language (2017)
- Writing a scientific paper; GSLS (2021)

Scientific integrity/ethics in science activities (0.3 ECTS)

- Workshop good academic practice; NIOO-KNAW (2020)

PE&RC Annual meetings, seminars and the PE&RC weekend (0.9 ECTS)

- PE&RC Last years weekend (2020)
- PE&RC Day (2021)

Discussion groups / local seminars or scientific meetings (6.9 ECTS)

- Silviculture science discussion at NFU (2017-2019)
- Microbial ecology seminar at NIOO (2020)
- Kuramae's discussion group (2020)
- Bioinformatic discussion group (2020)
- Wageningen Evolution & Ecology Seminar (WEES) and workshop (2021)

International symposia, workshops and conferences (1.8 ECTS)

- 6th International Conference on Nitrification and Related Processes (ICoN6); Xiamen, China (2019)
- A workshop on the Soil Microbial Science; Nanjing, China (2019)

Societally relevant exposure (1.5 ECTS)

- Summer social investigation on the ecological environment of Wuzhen, Zhejiang, China (2018)

Supervision of MSc students (3 ECTS)

- Effects of PGPR on nutrient uptake, plant growth, and bioactive compound accumulation in *Cyclocarya paliurus* (Batal.) Iljinskaja
- Bio-fertilization form and level affect the phytochemical responses and morphology of *Cyclocarya paliurus* (Batal.) Iljinskaja under field conditions
- Responses of soil nutrient properties to PGPR inoculation: a soil incubation test