Effects of combined application of *Bacillus subtilis* and humate biostimulants on plant growth

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Author:
Nietos Hietanen

Supervisors:
Professor Ville-Petri Friman
PhD. Eeva Marttinen

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Author: Nietos Laura Elina Hietanen

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Abstract:

Climate change and biodiversity loss are undermining conditions for food production, presenting a challenge for developing ecological farming methods to improve declining fertility of the soil, while producing crops efficiently. Biostimulants are much researched tool for improving plant health and productivity. Plant growth-promoting bacteria (PGPB) are commonly utilized as biostimulants and have been shown to increase plant biomasses, protect plants from pathogens and improve plant metabolism. Humic substances (HS) are a diverse group of large organic molecules commonly found in soil, also used as biostimulants. They stimulate root growth, induce metabolic changes in plants and enable plants to absorb micronutrients. Humic substances can be applied simultaneously with PGPB for utilizing their mutually reinforcing mechanisms on plant growth. Combination biostimulants have been shown to increase biomass, nutrient uptake and seed production in plants. Nevertheless, the knowledge on combination biostimulants and their exact impact mechanisms is still limited and inconsistent.

This study aimed to answer whether combining plant growth-promoting *Bacillus subtilis*-strains with humic substances would provide synergistic effects on crop growth. The combination biostimulants were prepared mixing either leonardite- or peat-based humate with two *Bacillus subtilis*-strains, respectively. Bacteria and humates were applied also as single compound biostimulants for comparison. Lettuce plants were treated repeatedly with the biostimulants and the effects on plant growth were measured as shoot and root weight and leaf chemical parameters.

In the first experiment, there were several interactions between bacteria and humate which increased lettuce shoot weight, root weight and flavonol production compared to bacterial biostimulants. Lettuce treated with combination biostimulant B1+H2 had also significantly larger root weights compared to water control group. In the second experiment, B. subtilis strain B1 as a single component biostimulant and combination treatment with B2+H2 increased significantly the shoot and root weights but synergistic features of combination treatments on plant growth were not consistent between the experiments. Therefore, these results suggest that lettuce response to combination biostimulants is likely influenced by environmental factors and might not be easily repeatable.

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Tiivistelmä:

Ilmastonmuutoksen ja biodiversiteettikadon vaarantaman ruoantuotannon turvaamiseksi on välttämätöntä kehittää työkaluja, jotka mahdollistavat tehokkaan sadonmuodostuksen elvyttämällä maaperän heikentynyttä kasvukuntoa. Biostimulantit ovat yksi paljon tutkittu työkalu kasvinsuojeluun ja satomäärien lisäämiseen. Kasvunedistäjäbakteerit ovat yleisiä biostimulantteja, jotka lisäävät kasvin biomassaa, suojaavat patogeeneiltä ja parantavat kasvin aineenvaihduntaa. Humusaineet ovat yleisiä maaperästä löytyviä orgaanisia molekyylejä, joita käytetään myös biostimulantteina. Nekin edistävät juurten kasvua, vaikuttavat kasvin aineenvaihduntaan ja auttavat kasvien ravinteiden otossa maaperästä. Humusaineita ja kasvunedistäjäbakteereja voidaan käyttää yhdessä, jolloin ne vahvistavat toistensa kasvia hyödyttäviä vaikutuksia. Yhdistelmäbiostimulanttien on osoitettu kasvattavan kasvin biomassaa ja siementuotantoa, sekä helpottavan ravinteiden ottoa. Tästä huolimatta kombinaatiobiostimulantit ja niiden toimintaperiaatteiden yksityiskohdat tunnetaan vielä huonosti.

Tämä tutkimus selvitti, aiheuttaako eri *Bacillus subtilis*-kasvunedistäjäbakteerien ja humusaineiden yhdistelmä synergistisiä vaikutuksia kasvin kasvuun. Yhdistelmäbiostimulantit valmistettiin joko leonardiitti- tai turvepohjaisista humusaineista ja kahdesta eri *B. subtilis*-kannasta. Biostimulantteja arvioitiin myös yksittäiskäsittelyinä. Käsittelyt toistettiin useasti salaatille ja vaikutukset mitattiin verson ja juuren painoina, sekä lehtimittauksin.

Ensimmäisessä kokeessa löydettiin monia vuorovaikutuksia bakteerien ja humusaineiden välillä, jotka vaikuttivat salaatin flavonolien tuotantoon, sekä versojen ja juurten painoon, verrattuna pelkkään bakteerikäsittelyyn. B1+H2-yhdistelmäbiostimulanttikäsittely kasvatti merkitsevästi juurten painoja myös verrattuna vesikontrolliin. Toisessa kokeessa yksittäiskäsittely *B. subtilis* B1-kannalla ja B2+H2-yhdistelmäbiostimulantilla kasvattivat merkittävästi verson ja juurten painoja, mutta synergistiset yhteisvaikutukset eivät silti olleet yhdenmukaisia kokeiden välillä. Tulokset viittaavat siihen, että ympäristöolosuhteet ohjaavat yhdistelmäbiostimulanttien vaikutusta salaatin kasvuun, jolloin tulokset eivät ole välttämättä toistettavissa.

1 Introduction

1.1 Agricultural challenges

Feeding the population of the world remains one of the central questions in our time. Intensive farming methods including heavy use of inorganic fertilizers contribute to declining condition of farming soils and climate instability (IPCC, 2019). One of the agricultural challenges is the loss of organic carbon in natural- and farming soils, leading to diminished plant productivity (Wiesmeier *et al.*, 2016; Wang *et al.*, 2022). Soil organic carbon (SOC) correlates with plant biomass, especially in nitrogen limited conditions (Ghaley *et al.*, 2018). SOC improves also water retention in soil, improving water supply for plants (Rawls *et al.*, 2003; Eden, Gerke and Houot, 2017). In addition to maintaining agricultural productivity, soil organic carbon is of great importance as a carbon pool, regulating atmospheric processes (Wiesmeier, Hübner and Kögel-Knabner, 2015). Previous research has claimed that even 25% of climate change solutions are linked to soil carbon, presenting a highly significant focus point for mitigating climate change (Bossio *et al.*, 2020). Farming techniques increasing SOC perform an essential role in carbon management (Tian *et al.*, 2024).

Contemporary agriculture is based on a high demand for artificial fertilizers, contributing to feeding the world's population by intensified production, as well as introducing environmental problems (Erisman *et al.*, 2008; Wang *et al.*, 2018; IPCC, 2019; Menegat, Ledo and Tirado, 2022). Production, transportation and the use of synthetic nitrogen fertilization are the sources of 10.6% of agricultural greenhouse gas (GHG) emissions, which translates into 2.1% of global emissions (Menegat, Ledo and Tirado, 2022). The efficiency of artificial nitrogen used today is very low, as a large proportion of the fertilizers become denitrified by micro-organisms into dinitrogen oxide, or alternatively are discharged into water bodies, causing eutrophication (Erisman *et al.*, 2008; Wang *et al.*, 2018; Menegat, Ledo and Tirado, 2022). Nitrogen fertilizing can be reduced by promotion of beneficial soil microbes, which render existing nitrogen accessible to plants (Santi, Bogusz and Franche, 2013; Kuan *et al.*, 2016).

Phosphorus is another element of significance, since it is also essential plant macro-nutrient and the second most limiting macronutrient after nitrogen (Alori, Glick and Babalola, 2017; Kumar, Kumar and Mohapatra, 2021; Zeng *et al.*, 2022). Phosphorus is

sufficiently abundant in soils, but in precipitated form, therefore unavailable for plants, which absorb phosphorus as H_2PO_4 or HPO_4^{-2} in soil solute (Berde, Rawool and Berde, 2021; Zeng *et al.*, 2022). In agriculture, the lack of insoluble phosphorus is mitigated by applying phosphorus fertilizers. A large part of the world's agriculture relays on phosphorus fertilizers derived from rock phosphate mining, a non-renewable, soon-to-be depleted natural resource (Cordell, Drangert and White, 2009). Fertilizer phosphate leaches to waterbodies and causes eutrophication, equivalent to nitrogen fertilizers (Cordell, Drangert and White, 2009). Substantial energy input is also required to produce phosphate fertilizer from phosphate rock. Additionally, the process generates fivefold amount of phosphogypsum, toxic and radioactive waste. Besides, a large proportion of the applied fertilizer phosphorus will be wasted, as phosphate readily chelates with metal cations in soil, rendering it inaccessible for plants (Zeng *et al.*, 2022).

Reducing excessive fertilization is required to lessen the environmental burden, but efficacy of food production needs to be maintained (Zeng *et al.*, 2022). With correct farming methods, the existing nutrients in soil can be rendered increasingly available for crop plants, decreasing the need for fertilizing while sustaining the yields (Kitir Sen, Kocaman and Aydemir, 2025). Promotion of plant growth-promoting microbes in rhizosphere is one viable approach for increasing plant access to nutrients.

1.2 Microbial role in soil nutrient condition

Carbon, nitrogen and phosphate are central components in climate and environmental change, as well as in agriculture (Cordell, Drangert and White, 2009; IPCC, 2019; Menegat, Ledo and Tirado, 2022). Transformations and cycling of carbon, nitrogen and phosphate are mostly driven by microbial activities in soil (Kallenbach, Frey and Grandy, 2016; Alori, Glick and Babalola, 2017; Zeng *et al.*, 2022; Zhang *et al.*, 2023). These biological mechanisms can be utilized for resource-efficient, ecological agriculture (Zeng *et al.*, 2022).

Soil organic carbon, the prerequisite of successful food production in fields, is a product of microbial activities (Kallenbach, Frey and Grandy, 2016; Ma *et al.*, 2018; Tao *et al.*, 2023). The origin of the organic carbon can be traced back to primary production of plants, following exudation and root turnover, as well as formation of detritus (Six *et al.*, 2006;

Tao *et al.*, 2023). Micro-organisms utilize these plant residues; they convert some of this carbon to CO_2 in their energy metabolism, and while doing so incorporate carbon into their biomass and extracellular compounds they excrete (Six *et al.*, 2006; Tao *et al.*, 2023).

Microbial exudates tend to form stable carbon compounds in contact with soil minerals (mineral-associated organic carbon: MAOC), creating recalcitrant carbon pools (Liang, Schimel and Jastrow, 2017; Liao *et al.*, 2023; Zhao *et al.*, 2025). Particulate organic carbon (POC) is labile, as it is not bound to minerals, and therefore available for microbial consumption (Liao *et al.*, 2023). Microbial biomass can be counted in labile carbon pool, as biomass is rapidly decomposed to carbon dioxide (Liao *et al.*, 2023). Nevertheless, living biomass transforms into necromass, which can form MAOC in appropriate conditions, and therefore is an essential contribution to SOC (Ma *et al.*, 2018; Liao *et al.*, 2023).

Nitrogen is often the limiting resource for plant growth (Moreau *et al.*, 2019). Bacterial nitrogen fixation is a substantial source of nitrogen in terrestrial ecosystems, and utilized in agriculture by including legumes in crop rotation (Szumigalski and Van Acker, 2006; Erisman *et al.*, 2007; Hu *et al.*, 2021; Granada Agudelo *et al.*, 2023a). Nitrogen fixating bacteria, diazotrophs, are distributed across multiple taxa, and found in diverse environments (Falkowski, Fenchel and Delong, 2008). Nodule-forming rhizobia are a well-studied and globally significant group of nitrogen fixators, comprised of Alpha Proteobacteria, like *Azorhizobium, Bradyrhizobium* and *Rhizobia*, and Beta Proteobacteria, like *Paraburkholderia* species (Mahmud *et al.*, 2020; Tang and Capela, 2020; Granada Agudelo *et al.*, 2023b). These rhizobia have dipartite lifestyle; they can exist as free-living saprophytes or as symbiotic partners of a plant, often dwelling inside a nodule, a specified, oxygen-selective plant structure for nitrogen fixation (Granada Agudelo *et al.*, 2023a). In addition, *Frankia* sp. of Actinomycetes develop nodule-forming endosymbiosis with non-legume, actinorhizal plants (Santi, Bogusz and Franche, 2013). Most of these plants are wood-stemmed trees or shrubs, like *Alnus*.

Furthermore, free-living rhizobia fixate nitrogen in soil and associate with plants (Smercina *et al.*, 2019). Known species include for example *Bacillus, Klebsiella, Streptomyces* and *Pseudomonas* (Iniguez, Dong and Triplett, 2004; Solanki *et al.*, 2019; Ait Bessai *et al.*, 2023). Compered to nodule-forming rhizobia, conditions for free-living

nitrogen fixation often involve more oxygenous environment and less carbon for energy source (Smercina *et al.*, 2019). Oxygen directly inhibits nitrogen fixation; thus, microbes need to convey the problem by forming isolating biofilm or utilizing anaerobic pores in the soil.

Microbial ammonification of detritus is alternative source of soluble nitrogen in soil (Grzyb, Wolna-Maruwka and Niewiadomska, 2021). Microbes decompose nitrogen-containing organic material, starting with enzymatic proteolysis, producing amino acids, urea and other nitrogen-rich compounds. Several *Bacillus* sp. and *Pseudomonas* sp. are known to exhibit proteolytic activities (Rahman, Basri and Salleh, 2003; Wang and and Cheng, 2017). Proteolysis is followed by ammonification, in which the products are further decomposed to inorganic ammonia and side products. Deamination occurs mainly extracellularly in the soil.

Insoluble phosphate can be solubilized by phosphate-solubilizing bacteria (PSB) (Alori, Glick and Babalola, 2017; Cherif-Silini *et al.*, 2021; Zeng *et al.*, 2022). Typical phosphate solubilizing bacteria include *Agrobacterium spp., and Bacillus circulans, B. megaterium, B. polymyxa, B. subtilis*, *Enterobacter spp.* and *Pseudomonas spp.* (Alori, Glick and Babalola, 2017; Cherif-Silini *et al.*, 2021). Prior to phosphate solubilizing, organic phosphorus compounds require decomposition with phosphatases, following the solubilizing of the released phosphorus (Li *et al.*, 2019; Zeng *et al.*, 2022). Phytases are specific group of phosphatases, capable of hydrolysis of organic phytate (Chen *et al.*, 2020; Saadi *et al.*, 2021). The enzyme is found for example in *Bacillus subtilis* and *B. licheniformis* (Tye *et al.*, 2002; Chen *et al.*, 2020; Saadi *et al.*, 2021). Secretion of low molecular organic acid is a common mechanism for releasing phosphorus from insoluble compounds (Zeng *et al.*, 2022). Other mechanisms include production of hydroxyl ions, protons and carbon dioxide to the soil matrix (Cherif-Silini *et al.*, 2021). For example, *Nitrobacteria* and *Thiobacillus* produce hydrogen sulfide for solubilization of phosphorus (Zeng *et al.*, 2022). Secretion of these acidic compounds lowers the pH in soil solute, releasing phosphorus (Cherif-Silini *et al.*, 2021).

1.3 Biostimulants as plant growth promoters

Plants and microbes interact with each other, either by developing neutral relationships, benefiting mutually or one-sided from each other's presence, or establishing an

antagonistic relationship where they exert negative effects on each other (Ahmad, Ahmad and Khan, 2008; Sasse, Martinoia and Northen, 2018; Oubohssaine, Rabeh and Hnini, 2025). The interactions include nutrient transformations, and cooperation-induced resistance to pathogens and other stress conditions (du Jardin, 2015; Fiodor, Singh and Pranaw, 2021; Oubohssaine, Rabeh and Hnini, 2025). Plant-growth promoting microbes appear commonly in soil, but their count among the rhizosphere microflora is too limited to offer the best possible benefit for plants (Berde, Rawool and Berde, 2021). These naturally occurring relationships can be utilized as crop-plant applicable biostimulants.

Biostimulants are non-fertilizer substances or microbes applied to crop plants in order to improve their growth and physical properties (du Jardin, 2015; Drobek, Frąc and Cybulska, 2019). Biostimulants can enhance crop nutrient intake, stress tolerance and crop quality, as well as induce the formation of soil organic content. Du Jardin has defined biostimulants as any kind of substance or microbe, which is applied to plants for increasing nutrition conditions, stress tolerance and crop quality traits (du Jardin, 2015).

Common types of biostimulants comprise micro-organisms, humic substances, protein hydrolysates, seaweed extracts and botanicals, chitosans, and inorganic compounds such as silicates (du Jardin, 2015; Drobek, Frąc and Cybulska, 2019). Their contribution to plant growth and health, as well as mechanisms of action are diverse. The ecological prospects, efficiency and economic factors need to be assed individually. To answer the challenges of the contemporary agriculture, the biostimulant need to be economically feasible, environmentally produced and agriculturally effective (Drobek, Frąc and Cybulska, 2019).

1.3.1 Microbial biostimulants

Micro-organisms utilized in biostimulants are principally free-living or endosymbiotic fungi and plant growth-promoting bacteria (PGPB) (du Jardin, 2015). Both of them share characteristics of enhancing the nutrient uptake of plants and protecting them from pathogens (du Jardin, 2015; Delaeter *et al.*, 2024). Microbial biostimulants of both fungal and bacterial origin can reduce the need for fertilizers (Adesemoye, Torbert and Kloepper, 2009).

Mycorrhiza is a well-known form of plant-fungus symbioses, where fungus and plant root form structural connection for cooperation, either in the shape of ectomycorrhiza (EM) or

arbuscular mycorrhiza (AM) (Bonfante and Genre, 2010; Oldroyd, 2013). Mycorrhizal fungi enhance nutrient and water absorption in plants, and in turn receive sugars from plants. This highly beneficial interaction is utilized in several biostimulant products globally, though technical issues remain with products' short shelf-life, variable effects, axenic cultivations, application method and requirements of AM as an obligate symbiont (Delaeter *et al.*, 2024; Du *et al.*, 2024). Though more familiar, filamentous fungi are not the only fungus cooperating with plants, since yeasts have also been found to enhance root growth, increase chlorophyll content of leaves and increase seedling development (Amprayn *et al.*, 2012; Fernandez-San Millan *et al.*, 2020). Yeast biostimulants are increasingly studied and commercial products are currently available (Campobenedetto *et al.*, 2021; Mannino *et al.*, 2022).

Plant growth promoting bacteria (PGPB) are a diverse group of bacteria enhancing plant growth (Drobek, Frąc and Cybulska, 2019). Common generalist PGPB species include for example *Bacillus spp.*, (Rekha *et al.*, 2007a; Arestova and Ryabchun, 2020; Katsenios *et al.*, 2022) *Pseudomonas spp*. and *Serratia spp.* (Rekha *et al.*, 2007a; Zhao *et al.*, 2018). Nodule-forming diazotrophic bacteria of *Rhizobium spp*. are sometimes understood as biofertilizers and distinguished from other PGPB, or included in the category (du Jardin, 2015; da Silva *et al.*, 2021).

Plants can attract beneficial bacteria in their vicinity by emitting volatile organic compounds (VOCs) (Schulz-Bohm *et al.*, 2018). VOCs are small, vaporizing molecules which diffuse easily in liquid or gas, and used especially for communication. Chemotaxis guides PGPB in motility towards the plants, which leads to root adhesion (Liu *et al.*, 2024). Reversible adhesion is followed by irreversible attachment to root surface and colony formation (Knights *et al.*, 2021). Some species establish endosymbiotic relationship with plants, and colonize plant interior tissue, either via root tips or lateral roots (Liu *et al.*, 2024). Bacteria need to suppress, avoid or tolerate plant immune response for successful colonization.

Biostimulant utilized PGPB often contribute to plant nutrient access by nitrogen fixation, phosphorus solubilization and production of chelating agents, siderophores, and organic acids for harvesting of micro-nutrient minerals like iron (Fe) and zinc (Zn) (Olanrewaju, Glick and Babalola, 2017; da Silva *et al.*, 2021). Many *Bacillus* species, such *B. subtilis* and

B. megaterium can solubilize phosphate, fixate nitrogen, produce ammonia and improve plant salinity tolerance (Ait Bessai *et al.*, 2023).

PGPB can directly enhance plant growth by producing plant hormones, such as auxin, mostly as indole-3-acetic acid (IAA), cytokinin and gibberellin (Olanrewaju, Glick and Babalola, 2017). IAA can increase shoot and root length and seed germination (Radhakrishnan and Lee, 2016), protect from pathogens and solubilize phosphate (LipkovÃi *et al.*, 2021). The compound is produced by many *Bacillus* species, including *Bacillus flexus, B. megaterium, B. methylotrophicus* and *B. subtilis* (LipkovÃi *et al.*, 2021; Ait Bessai *et al.*, 2023).

Many PGPB, including *Bacillus* sp. can produce plant defense compounds such as chitinase, lipase, protease and acetoin (Ryu *et al.*, 2003; Ait Bessai *et al.*, 2023). Acetoin (3-hydroxy-2-butanone) and 2,3-butanediol have been shown to induce growth in Arabidopsis thaliana (Ryu *et al.*, 2003) and also enforce induced systemic resistance (ISR) in plants (Ryu *et al.*, 2004). Consequently, PGPB strengthen also plant resistance to pathogens and abiotic stress.

Although many soil bacteria species are known to express plant growth-promoting traits, it has been demonstrated that plant growth-promoting traits are not species dependent, but vary between strains (Ait Bessai *et al.*, 2023). Consequently, the effect of each strain on plant growth needs to be assessed individually.

1.3.2 Humic substances as biostimulants

Humic substances (HS) are complex organic molecules commonly found in soil (Nardi, Schiavon and Francioso, 2021). The chemical structure of humic substances is not precisely described, but common functional groups are known. These include many oxygen-rich compounds, such as carbonyl (-C=O), carboxyl (C(=O)OH) and connected to alcohols and phenols, hydroxyls (-OH).

Humic substances own their highly recalcitrant nature to high amount of aromatic compounds (García *et al.*, 2016; Nardi, Schiavon and Francioso, 2021). Unsubstituted C-aromatic and C-aliphatic groups are connected to high recalcitrance, and substituted C-aliphatic groups to labile behavior (García *et al.*, 2016). Their recalcitrant nature and

variable bioactive functional groups directs their capability to interact with plants and microbes and remain in soil resisting decomposition (da Silva *et al.*, 2021; Nardi, Schiavon and Francioso, 2021).

Humic substances stimulate root growth by participating in plant signaling pathways, mostly by inducing an auxin-like response in plants (Trevisan *et al.*, 2010; da Silva *et al.*, 2021). HS has been shown to induce DR5 reporter similarly to plant-hormone indole-3-acetic acid (IAA, auxin), which leads to lateral root formation (Trevisan *et al.*, 2010; Conselvan *et al.*, 2017). The enhancement of lateral root growth also provides openings for microbial colonization, benefiting plant-PGPB relationships (da Silva *et al.*, 2021). The origin of IAA in HS is likely due to the tendency of HS to form complexes with phyto-active compounds, produced by plants and microbes in rhizosphere (Nardi, Schiavon and Francioso, 2021). Complexes are formed with soil ions as well, enabling better absorption of micro-nutrients for plants (da Silva *et al.*, 2021). HS induces also plant root excretion of organic acids, which feed rhizosphere bacteria and therefore enhance bacterial colonization. Interactions with humic substances can assist with bacterial adhesion to the roots.

HS treatment of lettuce has been shown to induce phenylalanine ammonia-lyase (PAL) enzyme activity, which in turn leads to production of secondary metabolites (Oh, Trick and Rajashekar, 2009; Conselvan *et al.*, 2017). These include flavonols and other polyphenolic antioxidants. Flavonols alleviate abiotic environmental stress plants encounter, such as UV-radiation, drought and salinity, by modifying reactive oxygen species born in plant stress responses (Daryanavard *et al.*, 2023). Flavonols also regulate auxin transport root development, vegetative growth and pollen development. The antioxidant activity makes flavonols also important dietary compound for humans (Gervasi *et al.*, 2022). They have also several other health-improving features, such as antimicrobial properties and effectiveness against many chronic diseases.

1.3.3 Synergistic effects of plant growth promoting bacteria when combined with humates

Combined application of plant growth-promoting bacteria and humic substances aims to utilize the mutually reinforcing mechanisms of HS on PGPB and on plant growth. As HS supports bacterial growth, PGPB can benefit plants more effectively. Simultaneously, HS

directs plants to host bacteria more attentively, and hence a positive feedback loop is created. Studies have found that combined HS-PGPB biostimulants have increased crop biomass, nutrient uptake and other desired properties (Pishchik *et al.*, 2016; Ekin, 2020; Cozzolino *et al.*, 2021; da Silva *et al.*, 2021; Savarese *et al.*, 2022; Maková *et al.*, 2025).

Studies have shown that treating crop plants with simple combination biostimulants can affect plant metabolism (Pishchik *et al.*, 2016; Aguiar *et al.*, 2018; Cozzolino *et al.*, 2021). Combination of *Bacillus amyloliquefaciens* FZB42, humic acids (HA) and arbuscular mycorrhizal fungi, and similarly *Pseudomonas* with HA have been shown to increase phosphate uptake in maize (Cozzolino *et al.*, 2021). Likewise, combination treatment with humates and *Bacillus* sp. was demonstrated to achieve the highest chlorophyll and total nitrogen content in lettuce leaves (Pishchik *et al.*, 2016).

In a field experiment, it was found that combined application of leonardite-based HS and *Bacillus subtilis* and *B. megaterium* increased various plant size and sunflower productivity related measures, such as capitulum diameter, seed weight and number of seeds, more than treatment with only bacteria (Ekin, 2020). The combination effect was not detectable in plant height, where only single component treatments with bacteria or HS influenced the value significantly. In the study of Cozzolino et al. combination biostimulant with *B. amyloliquefaciens* and humate produced larger total biomass in maize compared to treatment with sole bacterial biostimulant, but similar treatment with *Pseudomonas* spp. and humates increased plant biomass similarly to single component bacterial treatment (Cozzolino *et al.*, 2021).

Using a mixture of several plant growth promoting microbe species in combination with humate products have been shown to increase harvests substantially (Savarese *et al.*, 2022; Maková *et al.*, 2025). Savarese et al. demonstrated how treating lettuce with combination of potassium humates (KH), compost tea (CT) and diverse microbe consortia inoculant (M+) improved plant biomass production and primary- and secondary metabolism most substantially (Savarese *et al.*, 2022). All treatments improved growth compared to control. The microbe inoculant contained ten microbial species, including several *Glomus sp., Bacillus sp.,* and other PGPB as well as fungi such as *Trichoderma* sp. Treating lettuce with combination of KH and CT, without microbial inoculum increased the most substantially the production of flavonols and other polyphenolic antioxidants.

In a study of Makova et al., strawberries were treated with a diverse combination biostimulant, which included comprehensive fertilizer applied with several *Bacillus* sp., *Azotobacter* sp. and five other PGPB species as well as humic and fulvic acids (Maková *et al.*, 2025). Treatment increased the strawberry yield by 95% compared to the control groups. The rather complex mixture of such is likely not feasible for each biostimulant product, but the experiment demonstrates the tremendous effect biostimulants can have on crop growth.

Simple combination stimulants, with one or a couple of plant growth promoting microbe and humate compound, appear to exceed single component microbe biostimulants by influencing plant metabolism and leaf chemical content, in addition to enhancing biomass (Pishchik *et al.*, 2016; Aguiar *et al.*, 2018; Cozzolino *et al.*, 2021). Complex combination biostimulants with several microbes and fertilizers are likely to deliver a more distinctive impact on plant biomass (Savarese *et al.*, 2022; Maková *et al.*, 2025).

The size of a minimal, effective microbial consortia which could increase crop sizes and yield remains to be determined, as well as the roles of each component demonstrated in multiple case studies (Niu *et al.*, 2017; Gopal and Gupta, 2019; Yang *et al.*, 2021). Effects are likely to vary according to the plant species, the used humate and microbial strains examined, as well as growth conditions. A substantial amount of research is hence still required to understand the mechanisms and possibilities of combination-biostimulants.

2 The aims of the study

In this study, the effect of combination biostimulants on crop growth was evaluated in greenhouse experiments. Two experiments were conducted with lettuce. In each experiment, the crops were treated with individual and combination biostimulants of all possible combinations and the effects on plant shoot and root weights and leaf chemical content measured. In the second experiment, environmental conditions were altered slightly to test the robustness of biostimulants and humates. The biostimulants studied were two distinct, plant-growth promoting *Bacillus subtilis*-strains, and leonardite- and peat-based humate products respectively.

The research aimed to answer whether the combined application of *Bacillus subtilis* and humate biostimulant exert synergistic effects on crop growth, and if the biostimulants tested induce different responses in plants. The combination of *Bacillus subtilis*-inoculant and humate product were expected to improve biomass more than the components alone, based on the theoretical complementary features of humate and bacteria combined. Moreover, chlorophyll content and nitrogen balance index were expected to be higher in combination biostimulant-treated lettuce, to reflect the expectedly better nutritional status. Finally, humate treatments and PGPB + humate combination treatments were expected to elevate flavonol content in lettuce, based on the ability of humates to stimulate antioxidant production in plants. The first experiment was repeated with alternative environmental conditions to assess the consistency of the biostimulant impact in plant growth.

3 Materials and methods

Experiments were set in greenhouse between September 2024 and March 2025 in Helsinki Finland. The microbial inoculations were prepared in laboratory conditions aseptically as described later in section 3.2.

3.1 Preparations of the biostimulants

3.1.1 Bacterial biostimulants

Two strains of *Bacillus subtilis* were used in experiments: a novel *Bacillus*-isolate from Finnish peatlands (NEOVA), called “Bacteria 1” (B1) and a commercial biostimulant product FUSVER® (Servalesa), called Bacteria 2 (B2). Bacterial mass from a LB plate cultivation was transferred into liquid LB broth (LB Broth, Difco™ Lennox, lot 2227852) in previous day to treatment, placed in a shaker (300 RPM) and incubated in ±28°C for approximately 24h (FOC-200E Cooled Incubator).

After incubation, the strains were centrifuged for 10 min in 5000 RPM (Eppendorf Centrifuge 5804 R), supernatant discarded, and pellet resuspended into corresponding amount of sterile milli-Q water.

Based on previous trials conducted in the laboratory, bacterial concentration was determined with OD600 value 0.5 when diluted to 1:10 (SpectroStar Nano, BMG Labtech). Adjusting the concentration based on OD-value was later found to be insufficient for achieving the desired concentration of 10^7, and therefore the OD value was first raised to 0.75, followed by adjusting the concentration with steady incubation time of 22-24h incubations in ±28°C, resulting in approx. 10^7 concentrations, which was ensured by conducting CFU counts on LB plates (LB agar, Difco™ Lennox, lot 3272497) Bacterial concentrations ranged from 10^5 to 10^7 in Lettuce 1 experiment and from 10^6 to 10^9 in Lettuce 2 experiment (Table 1).

Table 1: Bacterial concentrations of inoculants based on CFU counts

Experiment	Date applied	Strain	OD 600	CFU
Lettuce 1	6.11.2024	B1	0.53	$1 \cdot 10^5$
		B2	0.55	$2 \cdot 10^5$
	13.11.2024	B1	0.42	$2 \cdot 10^7$
		B2	0.42	$5 \cdot 10^6$
	20.11.2024	B1	0.75	$1 \cdot 10^6$
		B2	0.71	$1.9 \cdot 10^5$
	27.11.2024	B1	-	$8 \cdot 10^7$
		B2	-	$6 \cdot 10^7$
Lettuce 2	21.2.2025	B1	-	$3 \cdot 10^7$
		B2	-	$2 \cdot 10^7$
	28.2.2025	B1	-	$1.8 \cdot 10^9$
		B2	-	$2 \cdot 10^8$
	7.3.2025	B1	-	$1.3 \cdot 10^7$
		B2	-	$1.7 \cdot 10^6$
	14.3.2025	B1	-	$4 \cdot 10^7$
		B2	-	$2.9 \cdot 10^7$

3.1.2 Humate biostimulants

Humate biostimulants were biostimulant were peat-based concentrate (NEOVA), called "Humate 1" (H1) and leonardite-based concentrate (CTA Humus, Meristem) called "Humate 2" (H2). Humus treatments corresponding 2 L / ha were prepared using reverse osmosis water and concentrates. Aliquot of 640 µl was pipetted into measuring bottle of 1000 ml, filling up the bottle to 1000 ml with reverse osmosis water. The solute was thoroughly mixed by overturning the bottle several times.

3.1.3 Applying the treatments

The treatments were amended by pipetting 1 ml of appropriate biostimulant or water control at the root of each plant, once per week. For plants receiving combination biostimulant treatment the overall amount of biostimulant was 2 ml / treatment.

3.2 Plant cultivations

Two similar lettuce experiments were conducted, referred as Lettuce 1 and Lettuce 2 experiments. In Lettuce 1 experiment, there were 36 replicant of each treatment and for Lettuce 2 experiment 18 replicants for each treatment.

Photoperiod with high-pressure luminaire lamps (400W) was set to 16h per day and temperature as ± 22°C in the Lettuce 1 experiment. In Lettuce 2 experiment, photoperiod was set to 15h per day and temperature as ±19°C for comparing the effects of biostimulants on plant growth in alternate environmental conditions.

Nutrient-limited soil was prepared by mixing thoroughly sand with granule size 0.5–1.2 mm (Weber, Sain-Gobain) with peat-based planting-mix (Kekkilä), in proportion of 95% v/v of sand and 5% planting-mix. Seeds of lettuce (*Lactuca sativa* 'Australische Gele') were sawn into 9 cm · 9 cm pots with 2 dl of prepared growth media 25.10.2024. Three seedlings were left in each pot and transplanted individually into same sized pots with 2dl media 26.11.2024. The experiment finished on 3.12.2024. Plants for Lettuce 2 experiment were sawn in 9 cm · 9 cm pots on 10.2.2025 and after germination one plant was left for each pot. The experiment finished on 22.3.2025.

Plants were assigned with random selection into arbitrary places in tables and moved three times per week, ensuring similar conditions for each individual pot.

All seedlings were fertilized with inorganic fertilizer mix for seedlings, containing total nitrogen (N) 19,0%, nitrate 7,3%, ammonium 1,8%, urea 10%, soluble phosphorus (P) 4,4%, soluble potassium (K) 20%, magnesium (Mg) 1,2%, boron (B) 0,025%, copper (Cu) EDTA-chelate 0,008%, iron (Fe) EDTA-chelate 0,17%, manganese (Mn) 0,08%, molybdenum (Mo) 0,005% and zinc (Zn) EDTA-chelate 0,012% (Kekkilä Taimilannoite, Kekkilä). The fertilizer was rationed based on the product instructions, mixed in irrigation water. The plants were fertilized thrice during Lettuce 1 experiment and once during Lettuce 2 experiment and irrigated sufficiently.

Table 2: Fertilizing of lettuces

Experiment	Date	Fertilizer solution per pot
Lettuce 1	15.11.2024	0.5 dl
	18.11.2024	0.5 dl
	22.11.2024	1 dl
Lettuce 2	13.3.2025	1 dl

3.3 Measuring the effects for plant growth

3.3.1 Plant weights

Effects of biostimulant treatments were assessed as measuring plant shoot weights and root weights in the end of the experiments. The shoots were measured directly after cutting the plant next to the first lateral root and weighed fresh using digital scale (EMAX, digital precision scale 0.01-200g, BIM8296). The roots were left on pots to dry overnight, to assist detaching soil from the roots. The following day each root was carefully picked from the pot, shaken gently to loosen soil from them and washed in plain water. Roots were left to dry on paper towel for a moment, pressed with a towel if needed to ensure similar dryness, and then weighed.

3.3.2 Leaf chemical parameters

The leaves of lettuce were measured in the end of the experiment, to assess whether the biostimulant treatments affected their metabolic status. Leaf absorbance was measured using Dualex (Metos®, Pessl Instruments) in the previous day to shoot weighing. Chlorophyll content (Chl) and flavonol index (Flav) were measured non-destructively. Chlorophyll content (Chl) indicates photosynthetic activity and general plant health status, and flavonol index (Flav) signals mostly of plant stress responses. Nitrogen balance index (NBI) is calculated based on chlorophyll and flavonol values to proportion nitrogen status with environmental situation. Each plant was measured three times, from the three youngest leaves when plausible. The average value was then calculated and used for further analysis. During Lettuce 1 experiment the measurements of leaf chemical content were performed on 2.12.2024 and during Lettuce 2 experiment on 22.3.2025.

3.4 Statistical analyses

Statistical effect of single biostimulants on plant growth were assessed using a one-way ANOVA in R, for each measured variable. Interactions of combination biostimulants were tested performing a two-way ANOVA in R for all measured data.

To meet the assumptions of two-way ANOVA, normal distribution of the data was analysed using Shapiro-Wilk's normality test, the homogeneity of variances by Levene's test and outliers identified. Based on the results, either parametric two-way ANOVA or non-parametric Welch's ANOVA was chosen.

Two-way ANOVA tests were followed by analysis of simple main effects with Bonferroni adjustment, either with overall error term or with separate error term, depending on the data meeting the assumptions. Multiple pair-wise comparisons were conducted to determine groups with demonstrated statistical significance.

One-way ANOVA or Welch's ANOVA, including related post hoc methods Tukey's HSD and Holm's method respectively, were conducted to all test groups to gain overall understanding of effectiveness of biostimulants in increasing weights or other measured values.

Figures demonstrating the results were created using ggplot2 in R. All statistical analyses were performed using R base and external packages car, dplyr, emmeans, forcats, ggplot2, ggpubr, ggsignif, ggtext, rstatix and tidyverse. OpenAI's ChatGPT (GPT-4 model) was utilized in resolving coding errors and formatting of results from statistical tests. Suggestions were critically assessed and manually implemented.

4 Results

4.1 Biostimulant effects on plant sizes

The effect of biostimulants on plant growth was assessed by measuring shoot and root weights of lettuce and comparing the groups to each other with statistical methods. In the Lettuce 1 experiment, significant differences in shoot or root weight were not found between the groups treated with individual biostimulants, B1 (*B. subtilis* NEOVA), B2 (*B. subtilis* Fusver, Servalesa), H1 (peat-based humate product, NEOVA) and H2 (leonardite-based humate product) (shoot $F_{4,175}$=1.199; P=0.313; root: $F_{4, 86.11}$ = 0.76; P = 0.552) (Figure 1a, b). In other words, single component biostimulants did not increase or decrease the plant weights in Lettuce 1 experiment.

Conversely, in Lettuce 2 experiment bacterial biostimulant B1 applied alone increased lettuce shoot weight ($F_{4,85}$=3.325; P=0.014) and root weight significantly ($F_{4,80}$=5.101; P<0.01) (Figure 1 c, d). The plant group treated with *B. subtilis* B1 were significantly heavier compared to the other test groups treated with single component biostimulants, regarding both shoot and root weight.

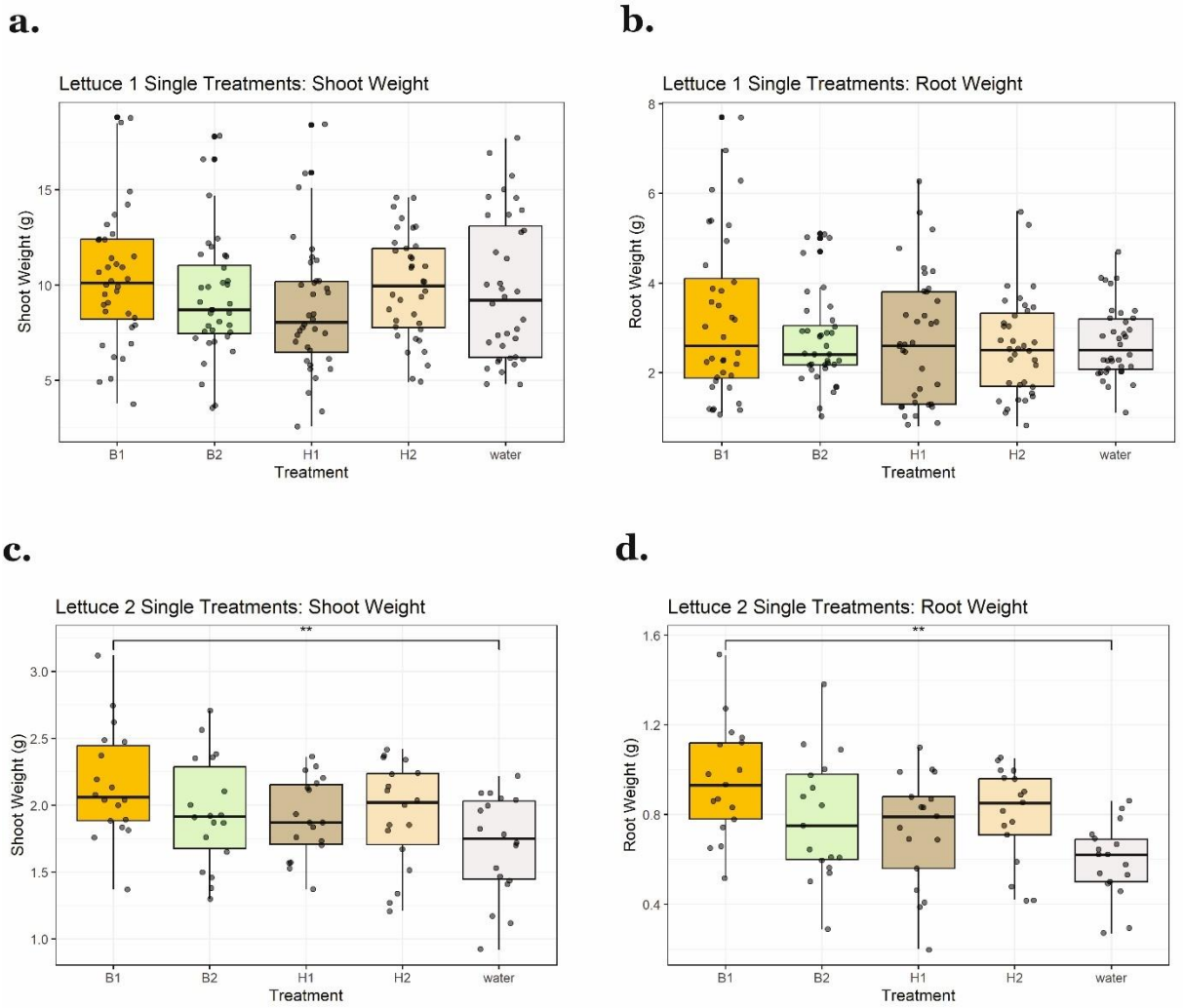

Figure 1: Comparative shoot and root weights (g) of lettuce in two independent experiments (Lettuce 1 and Lettuce 2) following application of single component biostimulants B.subtilis 'B114' (B1), B.subtilis 'Fusver' (B2), peat-based humate product 1 (H1) and leonardite-based humate product 2 (H2) a) Shoot weights of Lettuce 1 experiment b) Root weights of Lettuce 1 experiment c) Shoot weights of Lettuce 2 experiment d) Root weights of Lettuce 2 experiment

To answer the question whether bacterial and humate biostimulant demonstrate synergistic co-effects on plant biomass production, the interactions between bacteria and humate treatments affecting plant shoot and root weights were analysed.

In Lettuce 1 experiment it was found that combining bacteria and humate biostimulants altered plant shoot weights, compared to bacteria or humate applied individually, as a significant interaction between bacterial and humate treatments on shoot weight was found conducting two-way ANOVA ($F_{4,315} = 2.61$; $P < 0.05$, Figure 2a). Analysis of simple main effects of humate products for bacterial treatment was performed with statistical significance receiving a Bonferroni adjustment, revealing significant difference in plant shoot weight when humates were applied in combination with *Bacillus subtilis* B2 ($F_{2,315} = 4.18$; $P < 0.05$). The result was calculated using the overall error term of the two-way

ANOVA model. However, all the assumptions of two-way ANOVA were not met in the data distribution, hence the result was ensured calculating using separate error terms, resulting in the same outcome of humate shaping the impact of B2 on plant shoot weight ($F_{2,105}$ = 4.45; P < 0.05). The analyses were continued by multiple pair-wise comparisons to determine which humate groups in combination with bacteria resulted in significant differences regarding the shoot weight. It was found that only H1 and B2 combined demonstrated significant effect on shoot weight compared to B2 without humate (t_{315} = 2.88; P < 0.05). Differences between the other groups were non-significant in this test. To elaborate this result, one-way ANOVA was conducted for test groups treated with B2, H1, B2+H1 and water, showing significant differences between the groups ($F_{3,158}$ = 4.352; P < 0.01). Tukey's HSD supports the results of synergistic features of B2+H1 on shoot growth, since shoot weights of the group were significantly higher than B2 (diff = 2.24; P<0.05) and H1 (diff = 2.81; P<0.01). Differences to water control were non-significant within all test groups. In conclusion, peat-based humate product H1 applied in combination with *B. subtilis* B2 strain increased the shoot weights of lettuce in Lettuce 1 experiment compared to single component biostimulants, indicative of potential synergy (Figure 2 a). Nevertheless, when all the test groups were contrasted using one-way ANOVA and there appeared to be significant differences between the groups ($F_{8,315}$ = 2.396; P < 0.05), Tukey's HSD-test revealed B2+H1 did not differ significantly from the water control group (diff = 1.74; P=0.38) (Figure 2 b). Therefore, the shoot weight increasing effect of B2+H1 was not biologically significant in Lettuce 1 experiment.

Combined effects of bacteria-humate biostimulants on lettuce shoot sizes were not found in Lettuce 2 experiment with two-way ANOVA test ($F_{4,153}$ = 2.109; P = 0.082) (Figure 2c). In Lettuce 2 experiment, only B1 biostimulant alone resulted in statistically significant increase in shoot and root weight, as described earlier when examining the plant groups treated with single component biostimulants. Shoot weights of all the test groups were also compared to each other conducting one-way ANOVA. There were no statistically significant differences in shoot weight, calculated with one-way ANOVA ($F_{8,153}$ =1.976; P=0.0529). Since the P-value was on the borderline of significance threshold, Tukey's HSD was calculated. Based on Tukey's HSD, the shoot weights of B1 treated plants were larger compared to water control (diff= 0.46; P < 0.05), which supports the earlier result calculated without combination biostimulants (Figure 2d). Also, B2+H2 treated plant group had significantly larger shoot weights compared to water control (diff = 0.46; P <

0.05), suggesting bacteria-humate combination treatment might have increased shoot weights in additive manner, or by synergistic effects not detected with two-way ANOVA. The result was further investigated conducting one-way ANOVA only for test groups treated with B2+H2, B2, H2 and water, showing significant differences between the groups ($F_{3,68}$=4.001; P<0.05). Tukey's HSD confirmed the earlier result of B2+H2 treatment inducing larger weights compared to water control (diff = 0.46; P<0.01) but no significant differences between the single component treated plants and combination treated plants were found (B2+H2 and B2: diff = 0.21; P=0.39; B2+H2 and H2: diff = 0.23; P=0.34).

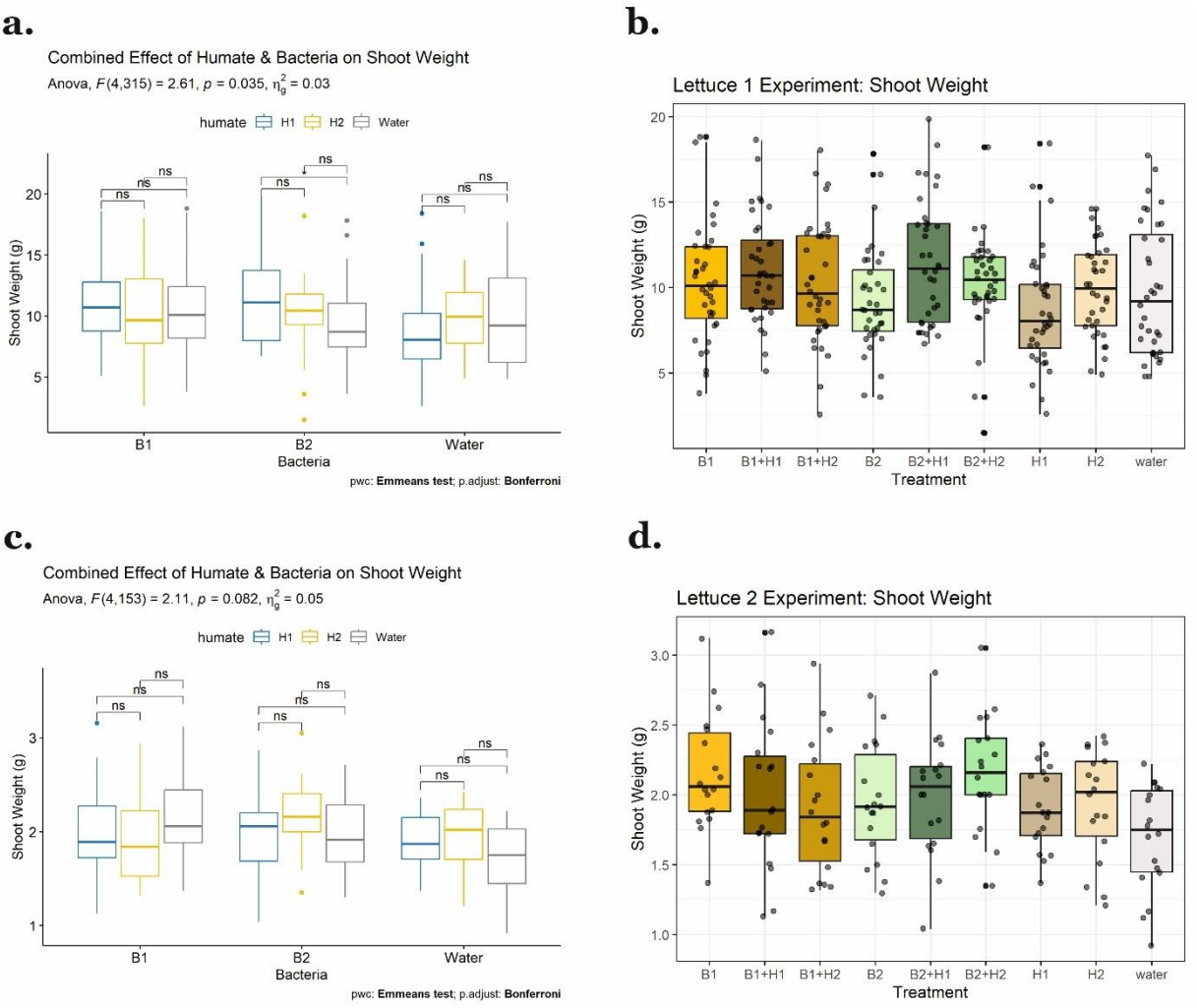

Figure 2: Comparative shoot weights (g) of lettuce in two independent experiments (Lettuce 1 and Lettuce 2) following application of combination biostimulants consisting of B.subtilis 'B114' (B1), B.subtilis 'Fusver' (B2), peat-based humate product 1 (H1) and leonardite-based humate product 2 (H2) a) Combined effects of humate and bacteria on shoot weights of Lettuce 1 experiment b) Shoot weights of Lettuce 1 experiment c) Combined effects of humate and bacteria on shoot weights of Lettuce 2 experiment d) Shoot weights of Lettuce 2 experiment

In Lettuce 1 experiment, similar but more considerable biomass-enhancing effect of combination biostimulants was observed regarding the root weight. Treatment with bacterial and humate biostimulants was found to increase root weight significantly ($F_{4,31}$=

6.253; P<0.0001). Analyses of simple main effects revealed combing either humate with bacteria made a significant increase on plant root weight (B1: $F_{2,315}$=12.0; P<0,0001; B2: $F_{2,315}$=3.27; P=0.039). Since three of the plant groups were not normally distributed (B1, B1 and B2+H1), comparisons were calculated using separate error rates, which confirmed the previous result. That is, combining humate with bacteria impacts significantly the effect of bacteria on root weight (B1: $F_{2,105}$=8.48; P<0,001; B2: $F_{2,105}$=4.03; P=0.021). Pair-wise comparisons using Bonferroni adjustment revealed that treatment combining H2 with B1 significantly enhanced the root weight compared to both B1 (t_{315} = 4.10, P < 0.001) and to H1 + B1 (t_{315} = 4.39; P < 0.001). Any differences regarding B2 with humates were no longer detectable, therefore the synergistic feature of B2 with humates on root weight were not proven. The results were further assessed by comparing the root weights of all groups to each other using non-parametric Welch's ANOVA ($F_{8,315}$ = 7.2027; P < 0.0001) and Holm's method to detect differences between the groups. This analysis confirmed that B1+H2 treated plants had significantly larger root weights compared to all other plant groups (compared to B2+H1 P= 0.04, P<0.0001 all others). In conclusion, only the combination of *B. subtilis* strain B1 with leonardite-based humate product H2 increased root sizes of lettuce synergistically in Lettuce 1 experiment (Figure 3a, b).

Combination of bacteria and humate treatment was found to affect significantly root weights in the Lettuce 2 experiment as well ($F_{4,144}$ = 4.700; P < 0.001). Analysis of simple main effects revealed humate influencing root weights when applied together with B1 ($F_{2,144}$ = 5.02; P < 0.01) and with water ($F_{2,144}$= 3.40; P < 0.05). The same groups were singled out with separate error rates. Pairwise comparisons revealed that H2 combined with B1 decreased root weights compared to B1 treatment alone (t_{144} = -3.10; P < 0.01) but increased the root weight when applied alone, compared to water (t_{144} = 2.56; P=0.034) (Figure 3c, d). The weights of all treatment groups were analysed with Welch's ANOVA ($F_{8,144}$ = 3.1142; P < 0.01) and Holms's test, which revealed that both B1 treated test group and B2+H2 treated group had significantly larger root weights compared to the water control group (P< 0.001 and P < 0.05, respectively). Impact of humate H2 on B2 root-growth promoting effectivity was not visible in pairwise comparisons, likely due to small differences between B2 single component biostimulant and B2+H2 treated plant group, similarly to H2+B2 treatment effect on shoot weights in the same experiment. Separate Welch's ANOVA for treatments including H2 and B2 and confirmed the result ($F_{3,64}$= 4.165; P < 0.01), but Holm's test found no differences between the single component

treated plants and combination treated plants, similarly to the test conducted for all treatments (water and B2H2: P < 0.01, B2+H2 and B2: P= 0.96, B2+H2 and H2: P = 0.96). In summary, in the Lettuce 2 experiment combining humate H2 with bacteria B1 decreased the root weights compared to application of bacteria B1 alone, contrary to the Lettuce 1 experiment. Applying B1 as a single component biostimulant increased the weights significantly (Figure 3d). Also, the plant group treated with B2+H2 was found to exhibit larger root mass than the water control group based on Welch's ANOVA analysis, but the result was not detectable when analysing co-effects of bacteria and humate products, possibly due smaller differences between B2 treated plant group and B2+H2 treated group.

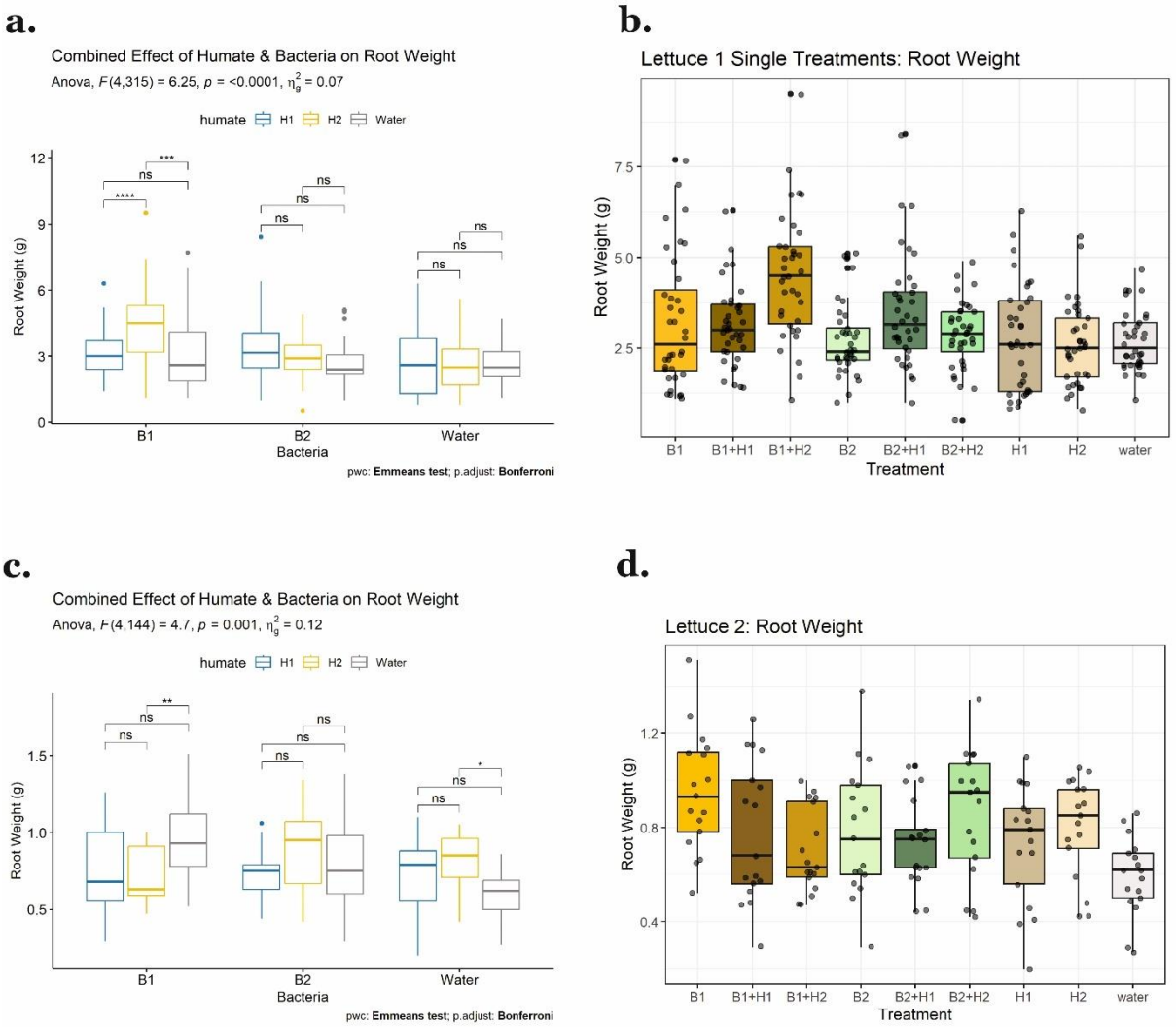

Figure 3: Comparative root weights (g) of lettuce in two independent experiments (Lettuce 1 and Lettuce 2) following application of combination biostimulants consisting of B.subtilis 'B114' (B1), B.subtilis 'Fusver' (B2), peat-based humate product 1 (H1) and leonardite-based humate product 2 (H2) a) Combined effects of humate and bacteria on root weights of Lettuce 1 experiment b) Root weights of Lettuce 1 experiment c) Combined effects of humate and bacteria on root weights of Lettuce 2 experiment d) Root weights of Lettuce 2 experiment

4.2 Biostimulant effects on leaf chemical content

In the end of the experiments, plant leaves were measured to investigate differences in the metabolic status between the test groups. Chlorophyll and flavonol levels were expected to increase in plants treated with PGPB or combination biostimulants. Humate treatments were assumed to increase flavonol content alone as well, based on earlier studies.

The analyses of leaf chemical content in Lettuce 1 experiment revealed that biostimulant treatments affected leaf flavonol content (Welch's ANOVA: $p < 0.001$), but not chlorophyll ($F_{4,86.2}=1.661$; $P = 0.17$) or NBI ($F_{4,85.9}=1.8492$; $P=0.13$). Plants treated with bacterial biostimulants exhibited significantly lower levels of flavonol content compared to water control, based on Games-Howell test ($P<0.001$) (Figure 4a). B1 treated plants had also significantly lower levels of flavonols compared to H2 treated plant group ($P = 0.049$). B2 treated plants demonstrated lower flavonol levels compared to both humates ($P < 0.05$). Conversely, in Lettuce 2 experiment there no statistically significant differences regarding the flavonol content in leaves ($F_{4,85} = 0.921$; $P = 0.456$) (Figure 4b). Contrary to expectation, bacterial biostimulants decreased flavonol levels in Lettuce 1 experiment and humate treatments did not increase flavonol content (Figure 4 a), and in Lettuce 2 experiment there were no differences between the groups in flavonol content (Figure 4 b).

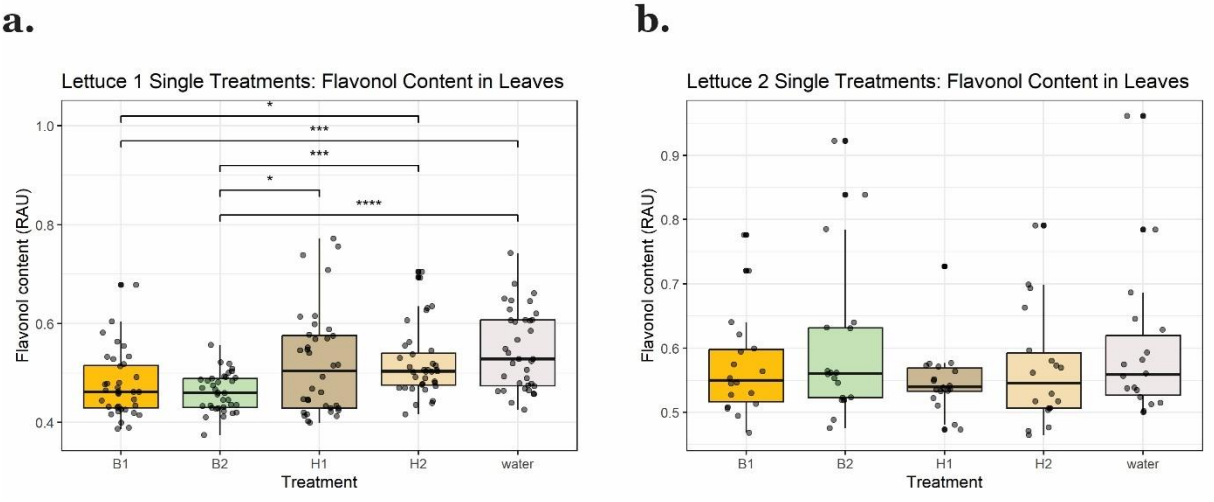

Figure 4: Comparative flavonol content (RAU) of lettuce in two independent experiments (Lettuce 1 and Lettuce 2) following application of single component biostimulants B.subtilis 'B114' (B1), B.subtilis 'Fusver' (B2), peat-based humate product 1 (H1) and leonardite-based humate product 2 (H2) a) Flavonol content of Lettuce 1 experiment b) Flavonol content of Lettuce 2 experiment

The analyses of combined effects showed that treatment with both bacterial and humate biostimulant increased the flavonol content in leaves during the Lettuce 1 experiment ($F_{4,297}$ = 23.47; P< 0.0001). Assessment of simple main effects of humates on bacteria revealed both bacterial strains with humates resulted in significant increase in flavonol content, calculated with model (B1 + humate: $F_{2.297}$ = 14.80; P < 0.0001, B2 + humate: $F_{2.297}$ = 14.30; P < 0.0001) or separate error rates (B1 + humate: $F_{2,105}$ = 13.1; P < 0.0001, B2 + humate: $F_{2,105}$ = 19.2; P < 0.0001). Multiple pair-wise comparison with Bonferroni adjustment showed combining B1 with H1 increases plant flavonol content more than in combination with H2 (t_{297} = 3.87; P < 0.001), and similarly with H2 compared to water (t_{297} = 5.06; P<0.0001). Combining B1 with H1 did not increase flavonol production in plants compared to plants treated with only B1 (t297 = 0.259; P=0.796). Combining B2 with either humate (H1 or H2) increased the flavonol production significantly (B2+H1: t_{297} = 5.00; P<0.0001, B2+H2: t_{297} = 4.13; P<0.0001) and the effect of humates compared to each other were indifferent (t_{297}= 2.07; P = 0.40). Based on Emmaens test, humate H2 increased flavonol content significantly compared to water control in combination tests where bacteria were included (t_{297} = 4.47; P<0.0001). No significant differences were found between bacterial treatments, therefore bacterial strain is not definitive in flavonol content changes (B1 vs B2: P>0.05).

For plants treated with *B. subtilis* B1, combining bacteria with humate H2 increased the flavonol content in plants, compared both to humate H1 and water (Figure 5a, b). For the plants treated with *B. subtilis* B2 adding either of humates increased flavonol content compared to bacteria without humates (Figure 5a, b). When comparing all the test groups using one-way ANOVA ($F_{8,297}$= 7.798; P < 0.0001), only B1 and B2 treated plant group differed from water control group significantly based on Tukey's HSD test (B1: diff = 0.072; P < 0.01, B2: diff = 0.86; P < 0.001), demonstrating significantly lower levels of flavonols compared to water control. One-way ANOVA and Tukey's HSD test did not show combination treated plant groups differing significantly from the control group, even the co-effect between bacteria and humate treatments was found in two-way ANOVA analysis. In the Lettuce 2 experiment, combination biostimulants made no statistically significant impact on flavonol content ($F_{4,153}$= 0.362; P = 0.835) (Figure 5c, d). Flavonol values were not changed in any way during Lettuce 2 experiment. In summary, during Lettuce 1 experiment, humate and bacteria demonstrated significant interaction, where inclusion of humate in bacterial inoculum increased plant flavonol production compared to sole

bacterial treatment. This was detected in B1 strain with H2 humate, and for B2 strain with both humates. In Lettuce 2 experiment, combination biostimulants did not alter flavonol levels of lettuce.

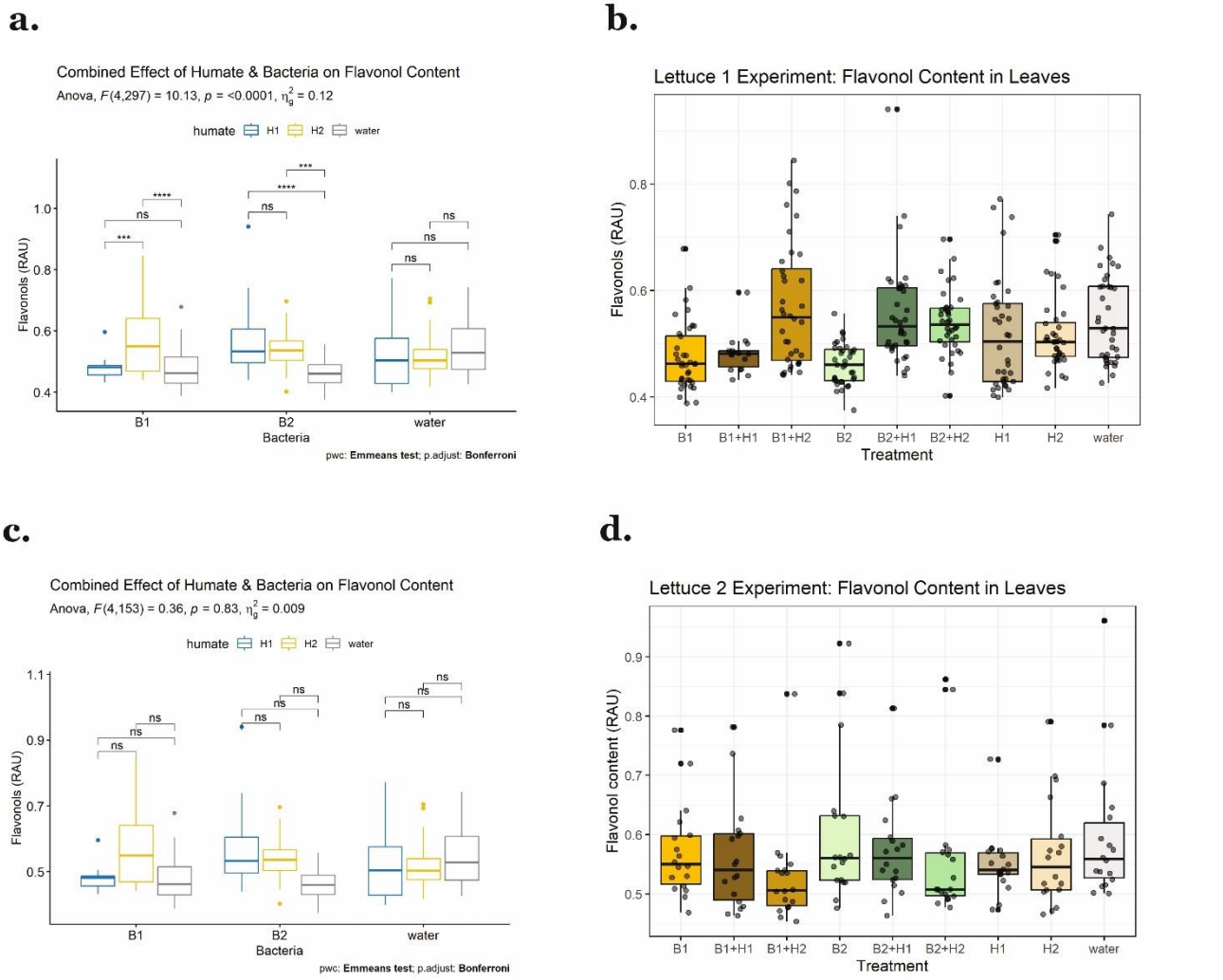

Figure 5: Comparative root weights (g) of lettuce in two independent experiments (Lettuce 1 and Lettuce 2) following application of combination biostimulants consisting of B.subtilis 'B114' (B1), B.subtilis 'Fusver' (B2), peat-based humate product 1 (H1) and leonardite-based humate product 2 (H2) a) Combined effects of humate and bacteria on flavonol content of Lettuce 1 experiment b) Flavonol content of Lettuce 1 experiment c) Combined effects of humate and bacteria on flavonol content of Lettuce 2 experiment d) Flavonol content of Lettuce 2 experiment

In contrary to Lettuce 1 experiment, significant changes were found in chlorophyll content (Chl) and nitrogen balanced index (NBI) in the Lettuce 2 experiment. The differences between plant groups treated with single component biostimulants were assessed first. Welch's ANOVA test displayed differences between those plant groups in their leaf chlorophyll content ($F_{4,41}$=4.81; P>0.01). Similar result was detectable in NBI ($F_{4,41}$ = 6.776; P< 0.001). Games-Howell test revealed *B. subtilis* B2 treatment increasing chlorophyll content compared to H2 (P=0.008) and conversely leonardite humate H2 to decrease chlorophyll (Chl) content compared to water treatment (P=0.036). The result was

highly similar regarding the NBI values, following the same trends (B2 vs H2: P=0.008; H2 vs water: P=0.036). Only the plants treated with H2 exhibited a significant difference in relation to the water control group, showing that H2 decreased the Chl and NBI values. The increase of Chl and NBI detected in B2 treatment was in relation to H2 treatment group and B2 treated test group did not differ from the control group. Contrary to the hypothesis, none of the individual component biostimulants increased chlorophyll or NBI content compared to water control treatment in Lettuce 2 experiment.

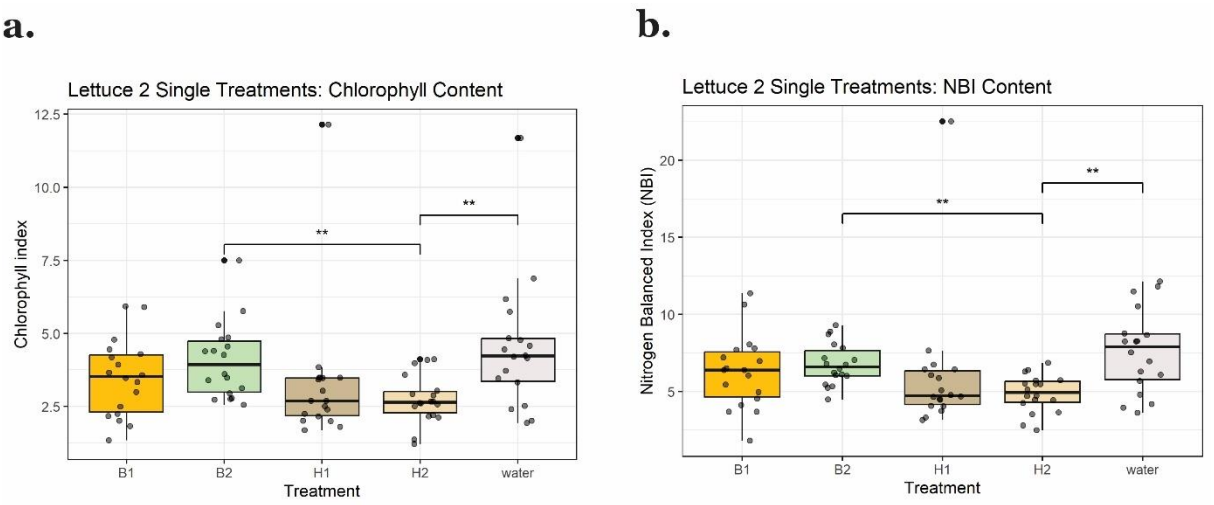

Figure 6: Comparative chlorophyll index and nitrogen balance index (NBI) of lettuce in two independent experiments (Lettuce 1 and Lettuce 2) following application of single component biostimulants B.subtilis 'B114' (B1), B.subtilis 'Fusver' (B2), peat-based humate product 1 (H1) and leonardite-based humate product 2 (H2) a) Chlorophyll index of Lettuce 1 experiment b) Nitrogen balance index of Lettuce 2 experiment

Combining humate with bacterial treatments appeared to influence chlorophyll content in leaves ($F_{4,153}$=2.482; $P < 0.05$). Analysing simple main effects suggested including humates with *B. subtilis* B2 would exhibit co-effect ($F_{2,153}$= 3.68; $P < 0.05$) on chlorophyll content. Similar result was received calculating with separate error rate ($F_{2,51}$= 3.78; P=0.03). Based on pair-wise comparisons (Bonferroni correction), combination treatments did not increase chlorophyll content ($P > 0.05$). Humate H2 treatment without bacteria decreased chlorophyll content compared to water control (t_{153} = 3.37; P<0.01; Figure 7 a, b). In NBI index, the results were the same, except significant difference was detected between B2+H1 and B2+H2 (Figure 7c, d). Therefore, the biostimulant treatments did not improve chlorophyll content or NBI value in lettuce in either of the experiments, either as single component biostimulants or as combination.

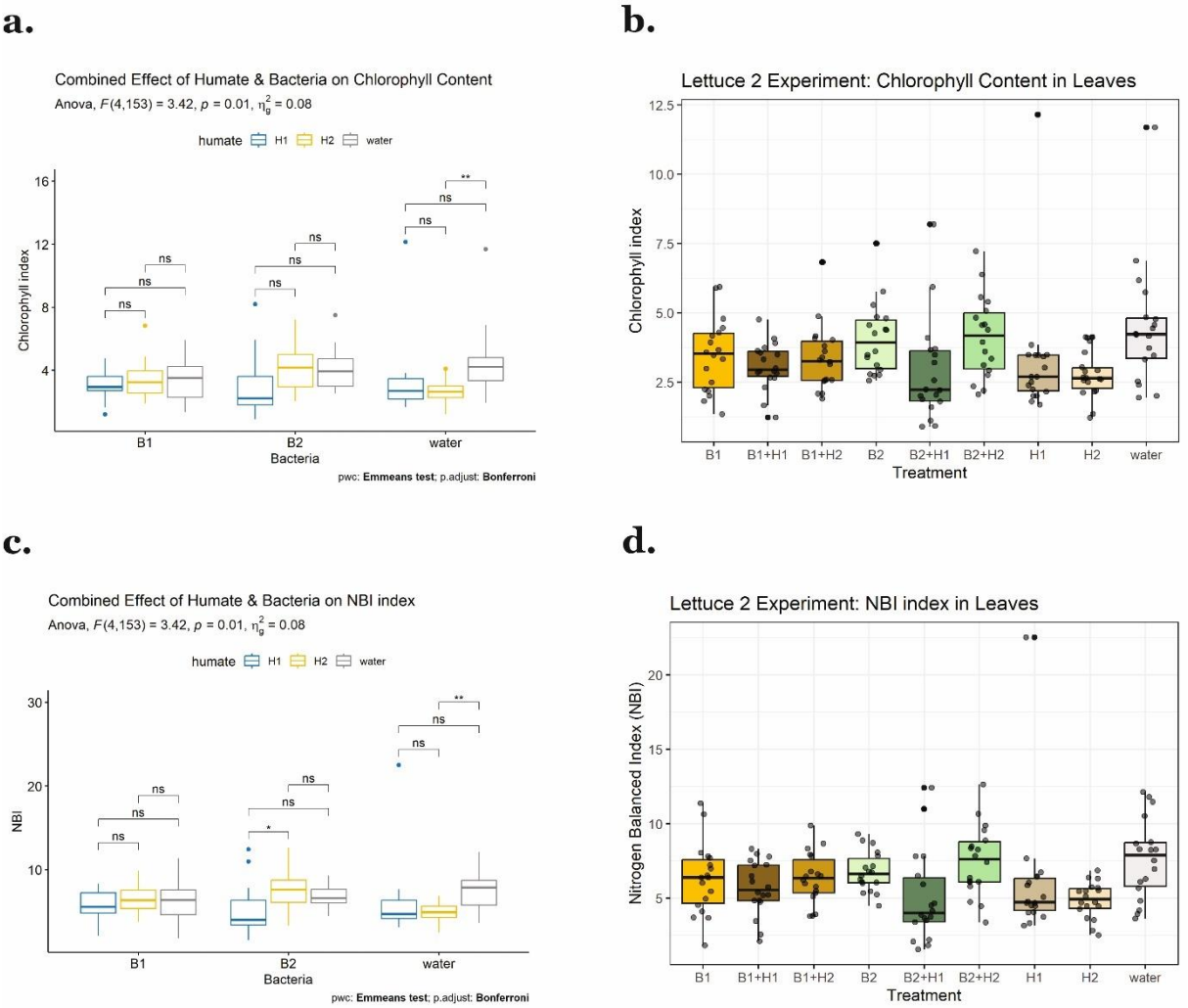

Figure 7: Comparative chlorophyll index and nitrogen balance index (NBI) of lettuce in two independent experiments (Lettuce 1 and Lettuce 2) following application of combination biostimulants consisting of B.subtilis 'B114' (B1), B.subtilis 'Fusver' (B2), peat-based humate product 1 (H1) and leonardite-based humate product 2 (H2) a) Combined effects of humate and bacteria on chlorophyll index of Lettuce 1 experiment b) Chlorophyll index of Lettuce 2 experiment c) Combined effects of humate and bacteria on nitrogen balance index of Lettuce 2 experiment d) Nitrogen balance index of Lettuce 2 experiment

5 Discussion

The aim of the study was to assess whether combining PGPB and humate into a combination biostimulant could deliver additional, synergistic features on crop growth compared to single component biostimulants. Combination biostimulants were expected to enhance lettuce biomass production more than PGPB inoculants without humates, based on studies with various plant species and alternative biostimulant combinations (Ekin, 2020; Cozzolino *et al.*, 2021; Savarese *et al.*, 2022; Othman *et al.*, 2024). Furthermore, combination biostimulants were expected to increase chlorophyll content in leaves, as shown in previous studies (Pishchik *et al.*, 2016; Aguiar *et al.*, 2018; Cozzolino *et al.*, 2021). Humate compounds were expected to enhance flavonol production in lettuce, as part of the combination biostimulant and as a single component (Oh, Trick and Rajashekar, 2009; Conselvan *et al.*, 2017). Two experiments were conducted with alternate environmental conditions. The results of the experiments were inconsistent with each other. In Lettuce 1 experiment, combining H2 humate with *B. subtilis* B2 enhanced PGPB's ability to support shoot growth, but shoot biomass was noticeably increased only in the Lettuce 2 experiment, when plants were treated with B1 single component biostimulant and B2+H2 combination biostimulant. In both experiments, lettuce root biomass was increased by B1-containing biostimulants, in the first experiment combined with humate H2, and in the second using B1 as a single component. Synergistic impacts of PGPB and humate combinations on plant biomass were detected confidently in Lettuce 1 experiment, where synergy of PGPB-HS biostimulants were found within some test groups in shoot and root weight variables and in flavonol content. In Lettuce 2 experiment, certain combination treatments increased lettuce weights compared to water control, but synergy was not detected with co-effect analysis, suggesting additive impact of bacteria-humate combination treatments.

Unlike expected, in Lettuce 1 experiment single component bacterial biostimulants did not increase plant weights. Commonly PGPB as single component biostimulant enhance plant biomass accumulation (Pishchik *et al.*, 2016; Ekin, 2020; Cozzolino *et al.*, 2021). In contrary to the results in Lettuce 1 experiment, application of B1 bacterial inoculant increased both shoot and root weights of lettuce in Lettuce 2 experiment. Impact of *B. subtilis* B1 strain on lettuce shoot weights is consistent with previous studies showing *B. subtilis* inoculations increasing lettuce weight (Oliveira *et al.*, 2023; Pagliarini *et al.*,

2023a), whereas single component treatment with B2 did not increase significantly neither shoot nor root weights in either of the experiments.

Combination of biostimulants *B. subtilis* B2 and peat-based H1 increased shoot weight compared to B2 without humate in Lettuce 1 experiment. Although the difference between B2+H1 and B2 treated plants in shoot weight was significant, signalling of the synergistic co-effect, the shoot weights of B2+H1 treated plant group did not differ significantly from the water control group. Therefore, the synergistic interaction between B2 and H1 was not sufficient to significantly increase lettuce shoot weight. The result is consistent with prior studies, as bacteria-humate combination biostimulants have been shown to both increase or not affect plant biomasses (Cozzolino *et al.*, 2021; Qiu *et al.*, 2025). In Lettuce 2 experiment interactions between bacteria and humate compounds were not found in co-effect analysis, but shoots of plant group treated with B2+H2 were significantly heavier compared to water control group, suggesting an additive impact. Plants treated with only B2 did not differ significantly from the water control group. The result is consistent with previous studies showing increase in plant biomasses by PGPB-humate combination biostimulants (Pishchik *et al.*, 2016; Cozzolino *et al.*, 2021; Othman *et al.*, 2024).

In Lettuce 1 experiment B1 combined with leonardite-based H2 increased root weights compared to B1 treated plant group, demonstrating a synergistic effect of combination biostimulant. Root weights of B1+H2 also differed significantly from control group and B1 treated plants in one-way ANOVA analysis, conforming the effectiveness of bacteria-humate synergy, in alignment with prior studies on simple PGPB-humate biostimulants (Rekha *et al.*, 2007b; Cozzolino *et al.*, 2021; Othman *et al.*, 2024). Quite the opposite was observed in Lettuce 2 experiment, where combining H2 with B1 was shown to decrease root weights, instead of increasing them. Similar results were observed in a study treating tomatoes with *B. subtilis* and humate, which produced smaller average fresh weights of tomato shoots compared to water control, although the difference did not reach statistical difference (Qiu *et al.*, 2025). Conversely, treatment with *B. subtilis* alone did not increase tomato fresh weights either, unlike in Lettuce 2 experiment. Compared to the water control group, B2+H2-treated group demonstrated significantly larger root weights, consistent with previous studies (Rekha *et al.*, 2007b; Cozzolino *et al.*, 2021; Othman *et al.*, 2024). The co-effect was not detected in two-way ANOVA analysis, possibly due to small differences between B2 and B2+H2 treated plants. The same was noted regarding the

shoot weights of B2+H2 treated plants like stated earlier. Even though the combination effects of B2 and H2 were not found, the result suggest that co-application of B2 and H2 improve plant growth consistently, but the effect is possibly additive. In both experiments, average shoot and root weights of B2+H2 treated plants were higher than average of B2 treated plants and mostly much higher than plants in water control group, in alignment with prior studies (Ekin, 2020; Cozzolino *et al.*, 2021; Othman *et al.*, 2024).

Humate compounds were expected to increase flavonol production in lettuce, both as part of the combination biostimulant and as a single component. In Lettuce 1, both bacterial treatments, B1 and B2 decreased flavonol content in plants compared to almost all other treatments. Humate treatments did not increase flavonol content compared to control, contrary to expectations. Flavonol concentration is often received as a measurement of plant's ability to encounter abiotic stress conditions, which was hypothesized also in this study (Oh, Trick and Rajashekar, 2009; Savarese *et al.*, 2022; Daryanavard *et al.*, 2023). Alternative explanation is that since flavonols accumulate in plants during stressful conditions, lack of flavonols signals of stress avoided. Since the plant sizes of groups treated with bacterial biostimulants were same or larger by average than control and plants signs of distress were not detected, it could be suggested that PGPB alleviate stress responses of plants or prevent occurring of stressful conditions.

Contrary mechanism was observed when combining humates with PGPB as combination biostimulant. In Lettuce 1 experiment, combination treatments increased significantly flavonol content in plants for most of the combination treated test groups (B1+H2, B2+H1, B2+H2). Only B1+H1 treated plants exhibited lower flavonol content compared to B1 treated counterparts, but there was no statistically significant difference between the groups. The results are somewhat consistent with prior study conducted with lettuce, showing how microbial inoculum (complex) combined with humate compound (green tea compost or potassium humates from leonardite) increased production of flavonols Quercetin 3-glucuronide, Quercetin 3-O-glucoside and of many other secondary metabolites more than the humates alone (Savarese *et al.*, 2022). Notably in Lettuce 1 experiment, the distribution of flavonol content in leaves seemingly followed the root weight distribution of the test groups, as plant groups with largest plants also had largest flavonol content. This suggests that flavonol concentration might signal plants experiencing nutrient deficiency stress for growing faster in nutrient-limited soil than their

counterparts treated with other biostimulants (Daryanavard *et al.*, 2023). Alternatively, if all the test groups are expected to experience similar stress conditions and flavonol levels are interpreted as measurement of stress resilience, correlation of high flavonol content with higher plant weights might signal overall benefits of combination treatments in plant growth during Lettuce 1 experiment.

In Lettuce 2 experiment, there were no significant differences between the groups in chlorophyll content, nitrogen balance index or flavonol index. The differences between the two experiments could be explained by different phenological growth state of the plants in the time of measurement. Even though the plants were cultivated approximately the same time (~5 weeks) the plants in Lettuce 1 experiment were significantly larger than plants in Lettuce 2 experiment. In Lettuce 1 experiment, the average shoot size was 10.1 g and root size 3.1 g. In Lettuce 2 experiment, they were 2,0 g for shoot and 0.8 g for root. The vast difference between the experiments was likely the outcome of different environmental growth conditions. Timing of bioinoculant application have been extensively studied in the perspective of how early colonizers define rhizosphere microbiome, but differentiating responses to same biostimulant during different growth phases of a plant remains underexplored (Kong *et al.*, 2025). Also, increasing the length of the photoperiod has been shown to increase flavonol production in plants (Fayezizadeh *et al.*, 2024). In the first experiment with higher flavonol content in plants, photoperiod was 16h in and in the second experiment slightly shorter, 15h. Experiments took place at different times of the year, which introduced variation in natural light. The differences in photoperiod length might partly explain differences in flavonol content between the experiments.

The two experiments were conducted in a similar manner, changes found in fertilizing, temperature, photoperiod length, bacterial concentration and replicate sizes. During Lettuce 1 experiment, plants received 2 dl of fertilizer per pot, and during Lettuce 2 experiment only 1 dl. Differences in nutrition likely contributed to plants growing larger during Lettuce 1 experiment and to the varying impact of biostimulants on plant growth parameters between the experiments. In a study conducted with lettuce, PGPB inoculum applied with fertilizers resulted in significantly larger plant biomass compared to both water control and PGPB consortia alone, between which no significant difference was found (Kitir Sen, Kocaman and Aydemir, 2025). Since PGPB supports plant growth largely by improving nutrient accessibility for plants, changing nutrient conditions in soil by

fertilizing with soluble nutrients is likely to influence effects of PGPB on plant growth (Zeng *et al.*, 2022).

The test plants were cultivated in different temperatures, in Lettuce 1 experiment ±22°C and in Lettuce 2 experiment ±17-19°C. The optimum temperature for *Bacillus subtilis* IAA production in liquid medium has been defined as +25°C (Wagi and Ahmed, 2019). In laboratory conditions, *B. subtilis* is often incubated in +37°C (Bolmanis *et al.*, 2024). Lettuce cannot be cultivated at +37°C but maintaining the temperature closer to +25°C might be better for bacterial growth. The temperature was closer to this optimum in the Lettuce 1 experiment, which could partly explain the differences between the experiments. Yet, *B. subtilis* appeared to flourish in colder temperature of +17-19°C, since the single component treatment with *B. subtilis* B1 produced the significantly largest plants in both shoot and root weights. Combination treatment with B2+H2 also resulted in significantly larger shoot and root weights compared to the control group. Hence, the temperature is not likely the ultimate explanation for the different results received from biostimulant treatments.

The inoculant concentrations varied between the two experiments. In Lettuce 1 experiment, the crops received four times biostimulants in consequential weeks, concentrations starting with 10^5 CFU/ml, increasing to 10^7 CFU/ml for the last treatment. In Lettuce 2 experiment, the concentration of inoculants remained approximately 10^7 during the whole experiment and all four doses. The concentration fluctuated from 10^7 to 10^9 based on CFU counts. CFU counts for *Bacillus subtilis* are relatively unreliable method for determining concentration since the species tend to swarm, confusing colony counting (Gorsuch *et al.*, 2019, 2020), but the concentration differences between the experiments are evident, since in Lettuce 1 experiment the 24h-inoculants were diluted based on OD 600-reading and in Lettuce 2 experiment the concentration of inoculants was regulated only with steady incubation times. The conflicting results in two similar experiments suggest that concentration of bacteria in biostimulant inoculant might have a directing role in the biomass-enhancing effect. It appears that combining humate compounds with *B. subtilis* in concentration of approximately 10^5 (CFU) mL^{-1} could be more effective in supporting plant growth compared to equal number of bacteria without humate biostimulant, underlining the synergistic combination effect. Conversely, when bacteria were applied in larger numbers (~10^9 (CFU) mL^{-1}), combined humate treatment did not

bring additional improvement to plant biomass. This phenomenon was most visible in combination treatments with *B. subtilis* B1 strain, B2 strain appeared to benefit from humates in both experiments. In a study with lettuce in aquaponic system, where *B. subtilis* inoculum concentrations ranged from 7.8×10^3, 15.6×10^3, 31.2×10^3, and 62.4×10^3 colony forming units (CFU) mL^{-1} of nutrient solution, which is equivalent to 3.9×10^7, 7.8×10^7, 1.56×10^8 and 3.12×10^8 (CFU) mL^{-1} per plant applied once, respectively (total CFU in reservoir of 100L/number of plants in the patch (20)) (Oliveira *et al.*, 2023). The highest concentration of inoculum, 62.4×10^3 colony forming units (CFU) mL^{-1}/100L (3.12×10^8 (CFU) mL^{-1} per plant) produced shoot weight equivalent to water control, and smaller root weights compared to water control. Largest shoot weights were produced by using 1.56×10^8 and 7.8×10^7 (CFU) mL^{-1} concentration per plant, and largest root weights with 7.8×10^7 (CFU) mL^{-1} concentration per plant. In summary, the largest concentration appeared to inhibit weight gain in lettuce, while smaller concentrations improved biomass accumulation. Conversely in many studies, concentration differences of inoculum did not alter the plant biomass enhancing effect of PGPB, or the effects were varying and non-linear (Escobar Diaz *et al.*, 2021; Pagliarini *et al.*, 2023b). Concentrations of *Bacillus subtilis* between 10^4 and 10^{10} CFU ml^{-1} have been show to result in similar plant shoot and root dry masses in cotton (Escobar Diaz *et al.*, 2021). In another study, concentrations of *Bacillus* VWC18 inoculant of 10^3 and 10^5 CFU·mL^{-1} increased fresh and dry weights of lettuce but concentrations of 10^7 and 10^9 CFU·mL^{-1} did not, though the experiment repeated with 10^3 and 10^9 CFU·mL^{-1} inoculants resulted in increased lettuce biomass in both concentrations (Pagliarini *et al.*, 2023b). The effect of inoculum concentration remains insufficient, and interactions of humate and inoculum concentration are even more underexplored. Assessing the effect of concentration would require a new experiment with standardized environmental factors.

Both experiments validated the plant growth-promoting traits of *Bacillus subtilis* and strain variability in the PGPB traits (Ait Bessai *et al.*, 2023). Combining *B. subtilis* B2 with humates gives relatively consistent results in both experiments, since combining B2 with humates improves the plant growth promoting effects of B2 in both experiments, within some test groups. The results were more complicated with B1 strain, where the two experiments delivered opposite results in plant growth when humate were included in the combination biostimulant. The relationship between *B. subtilis* B1 and different environmental factors needs to be analysed further with additional experiments.

The growth-promoting effects presented in this research are likely due to the nutrient harvesting traits of *B. subtilis*, which enabled plants to access the scarce nutrient resources in the poor soil, while humates supported bacterial growth and activated plant-growth via signaling pathways chemically (Zeng *et al.*, 2022). Plants might have accessed additional nitrogen also via *B. subtilis* nitrogen fixation and resisted pathogens with PGPB presence. The exact mechanisms of the tested biostimulants require further research.

Based on the two experiments conducted, the impact of combination biostimulants in lettuce growth is highly variable and possibly affected by inoculant concentration, fertilizing, temperature and photoperiod length. Since these conditions commonly variate in commercial agricultural settings, the combination biostimulants should be fine-tuned to achieve consistent results in diverse environmental conditions. The experiments could be repeated multiple times to estimate the average impact of the given biostimulant combinations on lettuce growth parameters.

The effects of the PGPB-humate combination biostimulants on crop chemical parameters could be researched in further detail, to assess whether and how consistently the treatments cause biochemical changes in plants. Combination biostimulant induced changes in flavonol content during the Lettuce 1 experiment suggest bacteria-humate combination biostimulants demonstrate prospects in enhancing plant metabolic capacities, as described in earlier research (Pishchik *et al.*, 2016; Aguiar *et al.*, 2018; Cozzolino *et al.*, 2021).

Additionally, resulting in more stable biostimulant microbial cohorts with several microbial species appear to be an efficient method to achieve larger biomass in crop plants (Santoyo *et al.*, 2021; Savarese *et al.*, 2022; Maková *et al.*, 2025). Since the combination biostimulants in the experiments delivered only limited and inconsistent improvements on lettuce biomass in this study, addition of another microbial species in the combination biostimulant could be beneficial in enhancing plant biomass accumulation. More complex biostimulant consortia could achieve better improvements on plant biomass accumulation and yield (Savarese *et al.*, 2022; Maková *et al.*, 2025). The minimal size and composition of an effective plant growth-promoting microbial consortia could be determined experimentally.

In conclusion, this research demonstrates the inconsistent impact of simple combination biostimulants on plant growth, but also the potential biostimulants entail as a solution for improving growth conditions in soil via environmentally sustainable way.

References

Adesemoye, A.O., Torbert, H.A. and Kloepper, J.W. (2009) 'Plant Growth-Promoting Rhizobacteria Allow Reduced Application Rates of Chemical Fertilizers', *Microbial Ecology*, 58(4), pp. 921–929. Available at: https://doi.org/10.1007/s00248-009-9531-y.

Aguiar, N.O. *et al.* (2018) 'Changes in metabolic profiling of sugarcane leaves induced by endophytic diazotrophic bacteria and humic acids', *PeerJ*, 6, p. e5445. Available at: https://doi.org/10.7717/peerj.5445.

Ahmad, F., Ahmad, I. and Khan, M.S. (2008) 'Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities', *Microbiological Research*, 163(2), pp. 173–181. Available at: https://doi.org/10.1016/j.micres.2006.04.001.

Ait Bessai, S. *et al.* (2023) 'The Plant Growth-Promoting Potential of Halotolerant Bacteria Is Not Phylogenetically Determined: Evidence from Two Bacillus megaterium Strains Isolated from Saline Soils Used to Grow Wheat', *Microorganisms*, 11(7), p. 1687. Available at: https://doi.org/10.3390/microorganisms11071687.

Alori, E.T., Glick, B.R. and Babalola, O.O. (2017) 'Microbial Phosphorus Solubilization and Its Potential for Use in Sustainable Agriculture', *Frontiers in Microbiology*, 8. Available at: https://doi.org/10.3389/fmicb.2017.00971.

Amprayn, K. *et al.* (2012) 'Plant growth promoting characteristics of soil yeast (*Candida tropicalis* HY) and its effectiveness for promoting rice growth', *Applied Soil Ecology*, 61, pp. 295–299. Available at: https://doi.org/10.1016/j.apsoil.2011.11.009.

Arestova, N. and Ryabchun, I. (2020) 'Influence of biostimulants on productivity and quality of grapes', *E3S Web of Conferences*, 210, p. 05001. Available at: https://doi.org/10.1051/e3sconf/202021005001.

Berde, C.P., Rawool, P. and Berde, V.B. (2021) 'Chapter 2 - Phosphate-solubilizing bacteria: Recent trends and applications in agriculture', in S. De Mandal and A.K. Passari (eds) *Recent Advancement in Microbial Biotechnology*. Academic Press, pp. 27–47. Available at: https://doi.org/10.1016/B978-0-12-822098-6.00004-5.

Bolmanis, E. *et al.* (2024) 'Pilot-scale production of Bacillus subtilis MSCL 897 spore biomass and antifungal secondary metabolites in a low-cost medium', *Biotechnology Letters*, 46(3), pp. 355–371. Available at: https://doi.org/10.1007/s10529-024-03481-4.

Bonfante, P. and Genre, A. (2010) 'Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis', *Nature Communications*, 1(1), p. 48. Available at: https://doi.org/10.1038/ncomms1046.

Bossio, D.A. *et al.* (2020) 'The role of soil carbon in natural climate solutions', *Nature Sustainability*, 3(5), pp. 391–398. Available at: https://doi.org/10.1038/s41893-020-0491-z.

Campobenedetto, C. *et al.* (2021) 'A Biostimulant Based on Seaweed (Ascophyllum nodosum and Laminaria digitata) and Yeast Extracts Mitigates Water Stress Effects on Tomato (Solanum lycopersicum L.)', *Agriculture*, 11(6), p. 557. Available at: https://doi.org/10.3390/agriculture11060557.

Chen, C. *et al.* (2020) 'Complete Genome Sequence of Bacillus sp. strain WR11, an Endophyte Isolated from Wheat Root Providing Genomic Insights into Its Plant Growth-Promoting Effects',

Molecular Plant-Microbe Interactions®, 33(7), pp. 876–879. Available at: https://doi.org/10.1094/MPMI-02-20-0030-A.

Cherif-Silini, H. *et al.* (2021) 'Tailoring Next Generation Plant Growth Promoting Microorganisms as Versatile Tools beyond Soil Desalinization: A Road Map towards Field Application', *Sustainability*, 13(8), p. 4422. Available at: https://doi.org/10.3390/su13084422.

Conselvan, G.B. *et al.* (2017) 'Biostimulant activity of humic substances extracted from leonardites', *Plant and Soil*, 420(1), pp. 119–134. Available at: https://doi.org/10.1007/s11104-017-3373-z.

Cordell, D., Drangert, J.-O. and White, S. (2009) 'The story of phosphorus: Global food security and food for thought', *Global Environmental Change*, 19(2), pp. 292–305. Available at: https://doi.org/10.1016/j.gloenvcha.2008.10.009.

Cozzolino, V. *et al.* (2021) 'Cooperation among phosphate-solubilizing bacteria, humic acids and arbuscular mycorrhizal fungi induces soil microbiome shifts and enhances plant nutrient uptake', *Chemical and Biological Technologies in Agriculture*, 8(1), p. 31. Available at: https://doi.org/10.1186/s40538-021-00230-x.

Daryanavard, H. *et al.* (2023) 'Flavonols modulate plant development, signaling, and stress responses', *Current Opinion in Plant Biology*, 72, p. 102350. Available at: https://doi.org/10.1016/j.pbi.2023.102350.

Delaeter, M. *et al.* (2024) 'Arbuscular Mycorrhizal Fungi as Biostimulant and Biocontrol Agents: A Review', *Microorganisms*, 12(7), p. 1281. Available at: https://doi.org/10.3390/microorganisms12071281.

Drobek, M., Frąc, M. and Cybulska, J. (2019) 'Plant Biostimulants: Importance of the Quality and Yield of Horticultural Crops and the Improvement of Plant Tolerance to Abiotic Stress—A Review', *Agronomy*, 9(6), p. 335. Available at: https://doi.org/10.3390/agronomy9060335.

Du, Y. *et al.* (2024) 'Arbuscular mycorrhizal fungi affect root morphology locally but not systemically through altering nutrient- and phytohormone-related gene expressions at low soil P level', *Plant and Soil*, pp. 1–18. Available at: https://doi.org/10.1007/s11104-024-07150-3.

Eden, M., Gerke, H.H. and Houot, S. (2017) 'Organic waste recycling in agriculture and related effects on soil water retention and plant available water: a review', *Agronomy for Sustainable Development*, 37(2), pp. 1–21. Available at: https://doi.org/10.1007/s13593-017-0419-9.

Ekin, Z. (2020) 'Co-Application of Humic Acid and Bacillus Strains Enhances Seed and Oil Yields by Mediating Nutrient Acquisition of Safflower (carthamus Tinctorius L.) Plants in a Semi-Arid Region', *APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH*, 18(1), pp. 1883–1900. Available at: https://doi.org/10.15666/aeer/1801_18831900.

Erisman, J.W. *et al.* (2007) 'Reduced nitrogen in ecology and the environment', *Environmental Pollution*, 150(1), pp. 140–149. Available at: https://doi.org/10.1016/j.envpol.2007.06.033.

Erisman, J.W. *et al.* (2008) 'How a century of ammonia synthesis changed the world', *Nature Geoscience*, 1(10), pp. 636–639. Available at: https://doi.org/10.1038/ngeo325.

Escobar Diaz, P.A. *et al.* (2021) 'Effect of Aspergillus and Bacillus Concentration on Cotton Growth Promotion', *Frontiers in Microbiology*, 12. Available at: https://doi.org/10.3389/fmicb.2021.737385.

Falkowski, P.G., Fenchel, T. and Delong, E.F. (2008) 'The Microbial Engines That Drive Earth's Biogeochemical Cycles', *Science*, 320(5879), pp. 1034–1039. Available at: https://doi.org/10.1126/science.1153213.

Fayezizadeh, M.R. *et al.* (2024) 'Variations in photoperiods and their impact on yield, photosynthesis and secondary metabolite production in basil microgreens', *BMC Plant Biology*, 24(1), pp. 1–13. Available at: https://doi.org/10.1186/s12870-024-05448-z.

Fernandez-San Millan, A. *et al.* (2020) 'Plant growth-promoting traits of yeasts isolated from Spanish vineyards: benefits for seedling development', *Microbiological Research*, 237, p. 126480. Available at: https://doi.org/10.1016/j.micres.2020.126480.

Fiodor, A., Singh, S. and Pranaw, K. (2021) 'The Contrivance of Plant Growth Promoting Microbes to Mitigate Climate Change Impact in Agriculture', *Microorganisms*, 9(9), p. 1841. Available at: https://doi.org/10.3390/microorganisms9091841.

García, A.C. *et al.* (2016) 'Structure-Property-Function Relationship in Humic Substances to Explain the Biological Activity in Plants', *Scientific Reports*, 6(1), p. 20798. Available at: https://doi.org/10.1038/srep20798.

Gervasi, T. *et al.* (2022) 'Biotechnological Applications and Health-Promoting Properties of Flavonols: An Updated View', *International Journal of Molecular Sciences*, 23(3), p. 1710. Available at: https://doi.org/10.3390/ijms23031710.

Ghaley, B.B. *et al.* (2018) 'Simulation of Soil Organic Carbon Effects on Long-Term Winter Wheat (Triticum aestivum) Production Under Varying Fertilizer Inputs', *Frontiers in Plant Science*, 9. Available at: https://doi.org/10.3389/fpls.2018.01158.

Gopal, M. and Gupta, A. (2019) 'Building plant microbiome vault: a future biotechnological resource', *Symbiosis*, 77(1), pp. 1–8. Available at: https://doi.org/10.1007/s13199-018-0574-z.

Gorsuch, J.P. *et al.* (2019) 'Enumeration of industrial *Bacillus* assemblages in commercial products with customized plate-counting assays', *Journal of Microbiological Methods*, 164, p. 105682. Available at: https://doi.org/10.1016/j.mimet.2019.105682.

Gorsuch, J.P. *et al.* (2020) 'Culture medium brand choice impacts colony swarming behavior among industrial *Bacillus* isolates and the accuracy of aerobic plate counts', *Journal of Microbiological Methods*, 172, p. 105891. Available at: https://doi.org/10.1016/j.mimet.2020.105891.

Granada Agudelo, M. *et al.* (2023a) 'The role of microbial interactions on rhizobial fitness', *Frontiers in Plant Science*, 14. Available at: https://doi.org/10.3389/fpls.2023.1277262.

Granada Agudelo, M. *et al.* (2023b) 'The role of microbial interactions on rhizobial fitness', *Frontiers in Plant Science*, 14. Available at: https://doi.org/10.3389/fpls.2023.1277262.

Grzyb, A., Wolna-Maruwka, A. and Niewiadomska, A. (2021) 'The Significance of Microbial Transformation of Nitrogen Compounds in the Light of Integrated Crop Management', *Agronomy*, 11(7), p. 1415. Available at: https://doi.org/10.3390/agronomy11071415.

Hu, H.-Y. *et al.* (2021) 'Nitrogen fixation and crop productivity enhancements co-driven by intercrop root exudates and key rhizosphere bacteria', *Journal of Applied Ecology*, 58(10), pp. 2243–2255. Available at: https://doi.org/10.1111/1365-2664.13964.

Iniguez, A.L., Dong, Y. and Triplett, E.W. (2004) 'Nitrogen Fixation in Wheat Provided by Klebsiella pneumoniae 342', *Molecular Plant-Microbe Interactions®*, 17(10), pp. 1078–1085. Available at: https://doi.org/10.1094/MPMI.2004.17.10.1078.

IPCC (2019) 'Summary for Policymakers. In: Climate Change and Land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems'. Available at: https://www.ipcc.ch/srccl/chapter/summary-for-policymakers/ (Accessed: 7 February 2025).

du Jardin, P. (2015) 'Plant biostimulants: Definition, concept, main categories and regulation', *Scientia Horticulturae*, 196, pp. 3–14. Available at: https://doi.org/10.1016/j.scienta.2015.09.021.

Kallenbach, C.M., Frey, S.D. and Grandy, A.S. (2016) 'Direct evidence for microbial-derived soil organic matter formation and its ecophysiological controls', *Nature Communications*, 7(1), p. 13630. Available at: https://doi.org/10.1038/ncomms13630.

Katsenios, N. *et al.* (2022) 'Assessment of plant growth promoting bacteria strains on growth, yield and quality of sweet corn', *Scientific Reports*, 12(1), p. 11598. Available at: https://doi.org/10.1038/s41598-022-16044-2.

Kitir Sen, N., Kocaman, A. and Aydemir, Ö.E. (2025) 'The role of chemical fertilizer reduction and different microbial inoculants on yield increase in lettuce cultivation', *BMC Plant Biology*, 25(1), pp. 1–11. Available at: https://doi.org/10.1186/s12870-025-06986-w.

Knights, H.E. *et al.* (2021) 'Deciphering bacterial mechanisms of root colonization', *Environmental Microbiology Reports*, 13(4), pp. 428–444. Available at: https://doi.org/10.1111/1758-2229.12934.

Kong, Z. *et al.* (2025) 'Priority effects of inoculation timing of plant growth-promoting microbial inoculants: role, mechanisms and perspectives', *Plant and Soil* [Preprint]. Available at: https://doi.org/10.1007/s11104-025-07291-z.

Kuan, K.B. *et al.* (2016) 'Plant Growth-Promoting Rhizobacteria Inoculation to Enhance Vegetative Growth, Nitrogen Fixation and Nitrogen Remobilisation of Maize under Greenhouse Conditions', *PLOS ONE*, 11(3), p. e0152478. Available at: https://doi.org/10.1371/journal.pone.0152478.

Kumar, Suresh, Kumar, Santosh and Mohapatra, T. (2021) 'Interaction Between Macro- and Micro-Nutrients in Plants', *Frontiers in Plant Science*, 12, p. 665583. Available at: https://doi.org/10.3389/fpls.2021.665583.

Li, C. *et al.* (2019) 'Environmental fungi and bacteria facilitate lecithin decomposition and the transformation of phosphorus to apatite', *Scientific Reports*, 9(1), p. 15291. Available at: https://doi.org/10.1038/s41598-019-51804-7.

Liang, C., Schimel, J.P. and Jastrow, J.D. (2017) 'The importance of anabolism in microbial control over soil carbon storage', *Nature Microbiology*, 2(8), pp. 1–6. Available at: https://doi.org/10.1038/nmicrobiol.2017.105.

Liao, J. *et al.* (2023) 'Divergent contribution of particulate and mineral-associated organic matter to soil carbon in grassland', *Journal of Environmental Management*, 344, p. 118536. Available at: https://doi.org/10.1016/j.jenvman.2023.118536.

LipkovÃi, N. *et al.* (2021) 'CHARACTERIZATION OF ENDOPHYTIC BACTERIA OF THE GENUS BACILLUS AND THEIR INFLUENCE ON THE GROWTH OF MAIZE (ZEA MAYS) IN VIVO', *Journal of microbiology, biotechnology and food sciences*, 10(5), pp. e3602–e3602. Available at: https://doi.org/10.15414/jmbfs.3602.

Liu, Y. *et al.* (2024) 'Root colonization by beneficial rhizobacteria', *FEMS Microbiology Reviews*, 48(1), p. fuad066. Available at: https://doi.org/10.1093/femsre/fuad066.

Ma, T. *et al.* (2018) 'Divergent accumulation of microbial necromass and plant lignin components in grassland soils', *Nature Communications*, 9(1), p. 3480. Available at: https://doi.org/10.1038/s41467-018-05891-1.

Mahmud, K. *et al.* (2020) 'Current Progress in Nitrogen Fixing Plants and Microbiome Research', *Plants*, 9(1), p. 97. Available at: https://doi.org/10.3390/plants9010097.

Maková, J. *et al.* (2025) 'Effect of Application of Nitrogen Fertilizer, Microbial and Humic Substance-Based Biostimulants on Soil Microbiological Properties During Strawberry (Fragaria × ananassa Duch.) Cultivation', *Horticulturae*, 11(2), p. 119. Available at: https://doi.org/10.3390/horticulturae11020119.

Mannino, G. *et al.* (2022) 'Changes in the Phytochemical Profile and Antioxidant Properties of Prunus persica Fruits after the Application of a Commercial Biostimulant Based on Seaweed and Yeast Extract', *International Journal of Molecular Sciences*, 23(24), p. 15911. Available at: https://doi.org/10.3390/ijms232415911.

Menegat, S., Ledo, A. and Tirado, R. (2022) 'Greenhouse gas emissions from global production and use of nitrogen synthetic fertilisers in agriculture', *Scientific Reports*, 12(1), p. 14490. Available at: https://doi.org/10.1038/s41598-022-18773-w.

Moreau, D. *et al.* (2019) 'A plant perspective on nitrogen cycling in the rhizosphere', *Functional Ecology*, 33(4), pp. 540–552. Available at: https://doi.org/10.1111/1365-2435.13303.

Nardi, S., Schiavon, M. and Francioso, O. (2021) 'Chemical Structure and Biological Activity of Humic Substances Define Their Role as Plant Growth Promoters', *Molecules*, 26(8), p. 2256. Available at: https://doi.org/10.3390/molecules26082256.

Niu, B. *et al.* (2017) 'Simplified and representative bacterial community of maize roots', *Proceedings of the National Academy of Sciences*, 114(12), pp. E2450–E2459. Available at: https://doi.org/10.1073/pnas.1616148114.

Oh, M.-M., Trick, H.N. and Rajashekar, C.B. (2009) 'Secondary metabolism and antioxidants are involved in environmental adaptation and stress tolerance in lettuce', *Journal of Plant Physiology*, 166(2), pp. 180–191. Available at: https://doi.org/10.1016/j.jplph.2008.04.015.

Olanrewaju, O.S., Glick, B.R. and Babalola, O.O. (2017) 'Mechanisms of action of plant growth promoting bacteria', *World Journal of Microbiology & Biotechnology*, 33(11), p. 197. Available at: https://doi.org/10.1007/s11274-017-2364-9.

Oldroyd, G.E.D. (2013) 'Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants', *Nature Reviews Microbiology*, 11(4), pp. 252–263. Available at: https://doi.org/10.1038/nrmicro2990.

Oliveira, C.E. da S. *et al.* (2023) 'Yield, nutrition, and leaf gas exchange of lettuce plants in a hydroponic system in response to Bacillus subtilis inoculation', *Frontiers in Plant Science*, 14. Available at: https://doi.org/10.3389/fpls.2023.1248044.

Othman, N.M. *et al.* (2024) 'Effectiveness of encapsulated plant growth-promoting bacteria, Acinetobacter sp. and organic supplements on the growth and nutrient contents enhancement in chilli plant', *Malaysian Journal of Microbiology*, 20(4), pp. 501–506.

Oubohssaine, M., Rabeh, K. and Hnini, M. (2025) 'Symbiosis vs pathogenesis in plants: Reflections and perspectives', *Microbial Pathogenesis*, 200, p. 107333. Available at: https://doi.org/10.1016/j.micpath.2025.107333.

Pagliarini, E. *et al.* (2023a) 'Yield and Nutraceutical Value of Lettuce and Basil Improved by a Microbial Inoculum in Greenhouse Experiments', *Plants*, 12(8), p. 1700. Available at: https://doi.org/10.3390/plants12081700.

Pagliarini, E. *et al.* (2023b) 'Yield and Nutraceutical Value of Lettuce and Basil Improved by a Microbial Inoculum in Greenhouse Experiments', *Plants*, 12(8), p. 1700. Available at: https://doi.org/10.3390/plants12081700.

Pishchik, V.N. *et al.* (2016) 'Estimation of synergistic effect of humic fertilizer and Bacillus subtilis on lettuce plants by reflectance measurements', *Journal of Plant Nutrition*, 39(8), pp. 1074–1086. Available at: https://doi.org/10.1080/01904167.2015.1061551.

Qiu, C. *et al.* (2025) 'The synergistic effects of humic acid, chitosan and Bacillus subtilis on tomato growth and against plant diseases', *Frontiers in Microbiology*, 16. Available at: https://doi.org/10.3389/fmicb.2025.1574765.

Radhakrishnan, R. and Lee, I.-J. (2016) 'Gibberellins producing *Bacillus methylotrophicus* KE2 supports plant growth and enhances nutritional metabolites and food values of lettuce', *Plant Physiology and Biochemistry*, 109, pp. 181–189. Available at: https://doi.org/10.1016/j.plaphy.2016.09.018.

Rahman, R., Basri, M. and Salleh, A.B. (2003) 'Thermostable alkaline protease from Bacillus stearothermophilus F1;: nutritional factors affecting protease production', *ANNALS OF MICROBIOLOGY*, 53(2), pp. 199–210.

Rawls, W.J. *et al.* (2003) 'Effect of soil organic carbon on soil water retention', *Geoderma*, 116(1), pp. 61–76. Available at: https://doi.org/10.1016/S0016-7061(03)00094-6.

Rekha, P.D. *et al.* (2007a) 'Effect of free and encapsulated *Pseudomonas putida* CC-FR2-4 and *Bacillus subtilis* CC-pg104 on plant growth under gnotobiotic conditions', *Bioresource Technology*, 98(2), pp. 447–451. Available at: https://doi.org/10.1016/j.biortech.2006.01.009.

Rekha, P.D. *et al.* (2007b) 'Effect of free and encapsulated *Pseudomonas putida* CC-FR2-4 and *Bacillus subtilis* CC-pg104 on plant growth under gnotobiotic conditions', *Bioresource Technology*, 98(2), pp. 447–451. Available at: https://doi.org/10.1016/j.biortech.2006.01.009.

Ryu, C.-M. *et al.* (2003) 'Bacterial Volatiles Promote Growth in Arabidopsis', *Proceedings of the National Academy of Sciences of the United States of America*, 100(8), pp. 4927–4932.

Ryu, C.-M. *et al.* (2004) 'Bacterial Volatiles Induce Systemic Resistance in Arabidopsis', *Plant Physiology*, 134(3), pp. 1017–1026. Available at: https://doi.org/10.1104/pp.103.026583.

Saadi, M.I. *et al.* (2021) 'Cloning of Bacillus subtilis phytase gene construct in Escherichia coli', *Iranian Journal of Microbiology* [Preprint]. Available at: https://doi.org/10.18502/ijm.v13i5.7433.

Santi, C., Bogusz, D. and Franche, C. (2013) 'Biological nitrogen fixation in non-legume plants', *Annals of Botany*, 111(5), pp. 743–767. Available at: https://doi.org/10.1093/aob/mct048.

Santoyo, G. *et al.* (2021) 'Plant Growth Stimulation by Microbial Consortia', *Agronomy*, 11(2), p. 219. Available at: https://doi.org/10.3390/agronomy11020219.

Sasse, J., Martinoia, E. and Northen, T. (2018) 'Feed Your Friends: Do Plant Exudates Shape the Root Microbiome?', *Trends in Plant Science*, 23(1), pp. 25–41. Available at: https://doi.org/10.1016/j.tplants.2017.09.003.

Savarese, C. *et al.* (2022) 'Combination of humic biostimulants with a microbial inoculum improves lettuce productivity, nutrient uptake, and primary and secondary metabolism', *Plant and Soil*, 481(1), pp. 285–314. Available at: https://doi.org/10.1007/s11104-022-05634-8.

Schulz-Bohm, K. *et al.* (2018) 'Calling from distance: attraction of soil bacteria by plant root volatiles', *The ISME Journal*, 12(5), pp. 1252–1262. Available at: https://doi.org/10.1038/s41396-017-0035-3.

da Silva, M.S.R. de A. *et al.* (2021) 'Humic Substances in Combination With Plant Growth-Promoting Bacteria as an Alternative for Sustainable Agriculture', *Frontiers in Microbiology*, 12. Available at: https://doi.org/10.3389/fmicb.2021.719653.

Six, J. *et al.* (2006) 'Bacterial and Fungal Contributions to Carbon Sequestration in Agroecosystems', *Soil Science Society of America Journal*, 70(2), pp. 555–569. Available at: https://doi.org/10.2136/sssaj2004.0347.

Smercina, D.N. *et al.* (2019) 'To Fix or Not To Fix: Controls on Free-Living Nitrogen Fixation in the Rhizosphere', *Applied and Environmental Microbiology*, 85(6), pp. e02546-18. Available at: https://doi.org/10.1128/AEM.02546-18.

Solanki, M.K. *et al.* (2019) 'Rhizospheric and endospheric diazotrophs mediated soil fertility intensification in sugarcane-legume intercropping systems', *Journal of Soils and Sediments*, 19(4), pp. 1911–1927. Available at: https://doi.org/10.1007/s11368-018-2156-3.

Szumigalski, A.R. and Van Acker, R.C. (2006) 'Nitrogen Yield and Land Use Efficiency in Annual Sole Crops and Intercrops', *Agronomy Journal*, 98(4), pp. 1030–1040. Available at: https://doi.org/10.2134/agronj2005.0277.

Tang, M. and Capela, D. (2020) 'Chapter Nine - Rhizobium diversity in the light of evolution', in P. Frendo, F. Frugier, and C. Masson-Boivin (eds) *Advances in Botanical Research*. Academic Press (Regulation of Nitrogen-Fixing Symbioses in Legumes), pp. 251–288. Available at: https://doi.org/10.1016/bs.abr.2019.09.006.

Tao, F. *et al.* (2023) 'Microbial carbon use efficiency promotes global soil carbon storage', *Nature*, 618(7967), pp. 981–985. Available at: https://doi.org/10.1038/s41586-023-06042-3.

Tian, J. *et al.* (2024) 'Microbially mediated mechanisms underlie soil carbon accrual by conservation agriculture under decade-long warming', *Nature Communications*, 15(1), p. 377. Available at: https://doi.org/10.1038/s41467-023-44647-4.

Trevisan, S. *et al.* (2010) 'Humic substances induce lateral root formation and expression of the early auxin-responsive IAA19 gene and DR5 synthetic element in Arabidopsis', *Plant Biology*, 12(4), pp. 604–614. Available at: https://doi.org/10.1111/j.1438-8677.2009.00248.x.

Tye, A. *et al.* (2002) 'Molecular cloning and the biochemical characterization of two novel phytases from B. subtilis 168 and B. licheniformis', *Applied Microbiology and Biotechnology*, 59(2), pp. 190–197. Available at: https://doi.org/10.1007/s00253-002-1033-5.

Wagi, S. and Ahmed, A. (2019) 'Bacillus spp.: potent microfactories of bacterial IAA', *PeerJ*, 7, p. e7258. Available at: https://doi.org/10.7717/peerj.7258.

Wang, M. *et al.* (2022) 'Global soil profiles indicate depth-dependent soil carbon losses under a warmer climate', *Nature Communications*, 13(1), p. 5514. Available at: https://doi.org/10.1038/s41467-022-33278-w.

Wang, S. and and Cheng, X. (2017) 'Changes in proteolytic bacteria in paddy soils in response to organic management', *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science*, 67(7), pp. 583–589. Available at: https://doi.org/10.1080/09064710.2017.1320422.

Wang, Yan *et al.* (2018) 'What could promote farmers to replace chemical fertilizers with organic fertilizers?', *Journal of Cleaner Production*, 199, pp. 882–890. Available at: https://doi.org/10.1016/j.jclepro.2018.07.222.

Wiesmeier, M. *et al.* (2016) 'Projected loss of soil organic carbon in temperate agricultural soils in the 21st century: effects of climate change and carbon input trends', *Scientific Reports*, 6(1), p. 32525. Available at: https://doi.org/10.1038/srep32525.

Wiesmeier, M., Hübner, R. and Kögel-Knabner, I. (2015) 'Stagnating crop yields: An overlooked risk for the carbon balance of agricultural soils?', *Science of The Total Environment*, 536, pp. 1045–1051. Available at: https://doi.org/10.1016/j.scitotenv.2015.07.064.

Yang, N. *et al.* (2021) 'Emergent bacterial community properties induce enhanced drought tolerance in Arabidopsis', *NPJ Biofilms and Microbiomes*, 7, p. 82. Available at: https://doi.org/10.1038/s41522-021-00253-0.

Zeng, Q. *et al.* (2022) 'Insight into soil nitrogen and phosphorus availability and agricultural sustainability by plant growth-promoting rhizobacteria', *Environmental Science and Pollution Research*, 29(30), pp. 45089–45106. Available at: https://doi.org/10.1007/s11356-022-20399-4.

Zhang, W. *et al.* (2023) 'Soil aggregate microstructure and microbial community structure mediate soil organic carbon accumulation: Evidence from one-year field experiment', *Geoderma*, 430, p. 116324. Available at: https://doi.org/10.1016/j.geoderma.2023.116324.

Zhao, Dan *et al.* (2018) 'Isolation and identification of bacteria from rhizosphere soil and their effect on plant growth promotion and root-knot nematode disease', *Biological Control*, 119, pp. 12–19. Available at: https://doi.org/10.1016/j.biocontrol.2018.01.004.

Zhao, G. *et al.* (2025) 'Soil minerals regulate soil organic carbon accumulation through glomalin-related soil protein along an elevation gradient in a mountain arid ecosystem', *Environmental Research*, 270, p. 121041. Available at: https://doi.org/10.1016/j.envres.2025.121041.