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Light-induced soil legacy alleviates continuous cropping adversity on Nicotiana benthamiana growth

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Research Article

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Abstract

Aims

Continuous cropping under artificial light is increasingly common in controlled-environment agriculture (CEA), yet it faces challenges like light stress and continuous cropping obstacles (CCO). This study explores how different light intensities shape the interactions among rhizosphere microbiota, soil properties, and allelochemicals during continuous cropping in CEA, with a focus on their roles in causing or alleviating CCO in plant.

Methods

We used *Nicotiana benthamiana* as the model plant and investigated its physiological responses, rhizosphere microbial dynamics, soil properties, and allelochemical profiles under different light intensities across three continuous cropping seasons.

Results

Low light significantly inhibited plant growth, but continuous cropping alone had minimal impact. In contrast, moderate light resulted in pronounced CCO, with high levels of allelochemicals (notably 2,4-ditert-butyl phenol and dimethylbenzene) disturbing microbial balance by suppressing beneficial bacteria (e.g., *Streptomyces*, *Massilia*), reducing functions like fermentation and nitrogen fixation, and increasing harmful processes such as denitrification. This disruption inhibited plant growth and intensified CCO. Soil legacy tests showed that removing negative legacies (allelochemicals and harmful microbes) eliminated CCO, highlighting their key role. Light-driven shifts in the microbiome increased by the third season. High light reduced yield decline between the second and third seasons, supporting more beneficial microbes. Under high-light conditions, lower allelochemical levels promoted beneficial microbes (e.g., *Pseudomonas, Flavobacterium*), stimulating plant hormone production and mitigating CCO.

Conclusions

Light intensity influences continuous cropping obstacles by regulating rhizosphere microbial structure and allelochemicals. These findings provide references for sustainable CAE management.

1. Introduction

Light stress, characterized by excessive or insufficient light intensity, altered light quality, or abnormal photoperiods, is a significant abiotic stressor that poses substantial challenges to plant growth and development (Fiorucci & Fankhauser, 2017; Shi et al., 2022; Yang et al., 2024). This issue is particularly

relevant in modern agricultural systems, where variations in light conditions due to seasonal changes, weather patterns, and artificial lighting in underground environments can all contribute to light stress (Roeber et al., 2021). Understanding how plants cope with light stress is crucial for optimizing agricultural productivity and ensuring food security.

In recent years, the role of plant-associated microorganisms in enhancing plant resilience to abiotic stresses has garnered increasing attention (Sandrini et al., 2022). Plant growth-promoting bacteria (PGPB) and arbuscular mycorrhizal (AM) fungi can improve plant tolerance to various environmental stresses by enhancing nutrient uptake, modulating phytohormone signaling, and mitigating stress damage (de Souza et al., 2015; Fadiji et al., 2023). These beneficial microbes form intimate interactions with plants, either as endophytes or through symbiotic associations, significantly influencing plant health and growth (Alsanius et al., 2019). Notably, the plant microbiome can also shape plant responses to light stress. For instance, certain microbial taxa can produce pigments or biofilms that protect plants from UV radiation and oxidative damage (Marín-Sanhueza et al., 2022; Vincent et al., 2022). Additionally, microbial modulation of phytohormone levels, such as abscisic acid (ABA), may help plants better adapt to high light conditions by regulating photosynthetic processes and stomatal closure (Ren et al., 2019). Compared to other environmental stresses, such as temperature and water, light stress cannot directly affect the soil or the microorganisms within it, highlighting the critical role of root exudates in the joint resistance of plants and microorganisms against light stress.

Root exudates act not only as microbial nutrients but also as signaling compounds that recruit beneficial microbes to assist plants in responding to abiotic and biotic stresses (Yuan et al., 2016). Previous studies have demonstrated that abiotic stresses such as drought and extreme temperature induce changes in root exudates, leading to shifts in soil microbial community composition and physicochemical properties. Furthermore, these shifts may persist beyond the immediate stress event, creating a "soil legacy" that can influence subsequent plant growth cycles (Bakker et al., 2018; Nguyen et al., 2018; Sun et al., 2019). For example, Chapelle et al. (2016) reported that in a soil suppressive to the fungal pathogen Rhizoctonia solani, the sugar beet rhizosphere microbiome was enriched for the bacterial families *Oxalobacteraceae*, *Burkholderiaceae*, *Sphingobacteriaceae*, and *Sphingomonadaceae*, while stress-related bacterial genes representing anti-fungal activities were specifically upregulated. Nguyen et al. (2018) found that long drought treatment before planting reduced the soil ammonia-oxidizing bacterial abundance and soil available nitrogen content, ultimately decreasing future plant productivity. Recent advances show that light stress can also alter the composition of root exudates, which in turn can influence the structure and function of rhizosphere microbial communities (Hodge et al., 1997; Hou et al., 2021; Martin et al., 2018; Wang et al., 2022). Hou et al. (2021) found that suboptimal light conditions significantly altered bacterial community structures in the rhizosphere, stimulating the growth of certain beneficial microbes that mitigated light-induced stress on plants. While the concept of soil legacy under drought or nutrient stress is well-documented, limited attention has been given to how light stress impacts the formation of soil legacy, particularly under continuous cropping systems.

Light intensity is one of the most common forms of light stress in controlled-environment agriculture, thus this study focuses on light intensity stress. Our study hypothesizes that, light intensity shapes soil microbial communities through influencing plant root exudates. The preserving of shaped soil microbial communities and the accumulation of root exudates contribute to the development of soil legacy and continuous cropping obstacles (CCO), respectively. We performed pot experiments to investigate (1) whether plants can recruit microbes to cope with light stress and CCO; (2) whether there is light-induced soil legacy left that can benefit the next generation of continue cropping plants. The results of the study may elucidate the mechanisms of interactions among rhizosphere microorganisms, soil and plants in continuous crop practices, and provide a theoretical basis for optimizing controlled environment agricultural systems and addressing challenges associated with CCO practices under artificial lighting conditions.

2. Materials and methods

2.1 Pots Experimental design

The experiment was conducted in the underground laboratory (3 meters below ground) of Sichuan University (30°37'44"N, 104°04'32"E). *Nicotiana benthamiana* was chosen as the plant material, and the nicotiana benthamiana seeds were kindly provided by the College of Life Sciences, Sichuan University. The soil used in this study was a mixture of PINDSTRUP peat soil (Pindstrup Mosebrug A/S, Ryomgaard, Denmark) and vermiculite (v:v, 3:1). The soil was with 124 mg/cm^3 bulk density, 250 μS/cm electrical conductivity, 48.19% organic carbon, 427 mg kg^{-1} available nitrogen, 60 mg kg^{-1} available potassium and a pH 5.63 at the beginning of the study.

The plant seeds were soaked in distilled water at 4℃ for 48h and then directly sown into plastic pots (10×10×9 cm) containing 70 g of dry soil. After germination, the plantlets were kept under light intensity of 150 μmol m^{-2} s^{-1}, 16 h of daylight and 8 h of dark period for 7 days before different light regimes were applied. The different light treatments included high light intensity (HL, 300 μmol m^{-2} s^{-1}), moderate light intensity (ML, 150 μmol m^{-2} s^{-1}) and low light intensity (LL, 30 μmol m^{-2} s^{-1}), all with the same photoperiod of 16 h. As long as the plants were transferred to different light treatments, the excessive plantlets were removed with only one plantlet left in each pot, and 15 ml of MS solution was added to each pot. Four weeks later, the aboveground plant parts were harvested and roots were removed from the soil. This process was repeated for three consecutive plant generations.

2.2 Plant agronomic traits and photosynthetic characteristics

The plant biomass was measured immediately after harvest. The plant height was measured from the soil surface to the top of a plant using a vernier caliper. The leaf area was determined by the coefficient methods (k = 0.60) (Bryan & Mullinix, 1992). Fresh weight (FW) of plant shoot and root was measured by an electronic analytical balance (OHAUS, PR124ZH/E, China), then plant samples were oven-dried (75℃)

until a constant mass was achieved to get the dry weights. The root-shoot ratio was determined by root dry weight dividing shoot dry weight.

Measurements on leaf photosynthetic characteristics were carried out two days before harvest. The parameters related to photosynthesis and gas exchange, including net photosynthetic rate (Pn), stomatal conductance (Cond), transient transpiration rate (Tr) and intercellular CO_2 concentration (Ci) of the fourth leaf of *N. benthamiana* were measured using the portable photosynthesis instrument (Yaxin-1101, Beijing, China).

2.3 Plant protective enzyme activities and hormone contents

After ground fresh leaf samples were added to 5 mL of phosphate buffer solution (PBS, 100 mM, pH = 7), the mixture was centrifuged for 10 minutes at 4000 rpm. The supernatant was used as the body enzymatic source, and the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were measured according to previous studies (Beyer & Fridovich, 1987; Keren-Keiserman et al., 2010; Aebi, 1984).

Fresh plant samples were used for the analysis of plant hormones. The plants were accurately weighed, and a phosphate-buffered saline (PBS) solution (9 times the sample weight, based on the weight/volume ratio) was added. The resulting 10% tissue homogenate was centrifuged at 2500 rpm for 10 minutes, and 50 μL of the supernatant was collected for hormone determination. Hormone contents, including indole-3-acetic acid (IAA), gibberellins (GA), cytokinins (CTK), and abscisic acid (ABA), were measured using enzyme-linked immunosorbent assay (ELISA) (Yang et al., 2002). Specific antibodies against IAA, GA, CTK, and ABA were employed, and the measurements were conducted using an ELISA reader, following the manufacturer's protocol.

2.4 Legacy soil slurry tests

A legacy soil slurry experiment was carried out to find the exact biological or chemical component in legacy soil that made a difference in plant productivity. Briefly, 100 g legacy soil of low light was thoroughly mixed with 1000mL deionized water and settling for 24h. Then soil slurry was collected and divided into three parts, one part was filtered through 0.45um filter polytetrafluoroethylene (PTFE) membrane, one part filtered through 0.22um PTFE membrane, and no treatment for the last part. Then 100ml of unfiltered soil slurry, 0.45um-filtered, 0.22um- filtered and deionized water (as control group) was added to a pot of initial soil (soil that never been planted before). Two days later, new *N. benthamiana* seeds were sowed and cultivated under low light intensity for another 35 days. Same operations were repeated for the legacy soils of moderate and high light. The experimental design is shown in Fig. 1.

2.5 Soil physicochemical analysis and allelochemicals

A cutting ring (2.8 cm in diameter and 2.5 cm in height) was used to sample the soil for soil bulk density measurement, and a fraction of bulk soil was air-dried and used for soil chemical analyses. Soil available nitrogen (N), available phosphorus (P), electrical conductivity and pH were measured according to Bao (2000).

The organic components in the unfiltered soil slurry and filtered soil slurry were analyzed by gas chromatography-mass spectrometry (GC-MS) with a modified method of Lisec et al. (2006). All samples were tested for their metabolite contents using a 7890A GC/5975C MS System (Agilent, USA). The injection, chromatography and mass spectrometer parameters were set accordingly. The peak identification, data baseline filtering and integration were processed using XCMS Software. Metabolite annotations were based on the retention time, mass-to-charge ratio (m/z) and mass spectral signature of all detectable ions. The samples were identified using the golm metabolome database (GMD) and NIST MS Search Program database. Moreover, the qualitative identification of 43 chemicals were confirmed using standard chemicals.

2.6 Rhizosphere soil 16S rRNA gene sequencing and bioinformatic analysis

When *N. benthamiana* was harvested from both pot experiments and legacy soil slurry tests, the rhizosphere soils were collected. Briefly, plant roots were gently removed from the soil and the rhizosphere soil (soil that adhered to plant root) were carefully scraped. The soil samples were immediately stored at -80°C prior to 16S rRNA sequence analysis.

Microbial DNA was extracted using the HiPure Soil DNA Kits (or HiPure Stool DNA Kits) (Magen, Guangzhou, China) according to the manufacturer's protocols. The 16S rDNA target region of the ribosomal RNA gene were amplified by PCR (94°C for 2 min), followed by 30 cycles at 98°C for 10 s, 62°C for 30 s (except for 16S V4: 55°C for 30 s), and 68°C for 30 s and a final extension at 68°C for 5 min. PCR reactions were performed in triplicate 50 μL mixture containing 5 μL of 10 × KOD Buffer, 5 μL of 2 mM dNTPs, 3 μL of 25 mM $MgSO_4$, 1.5 μL of each primer (10 μM), 1 μL of KOD Polymerase, and 100 ng of template DNA. Related PCR reagents were from TOYOBO, Japan. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster City, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (PE250) on an Illumina platform according to the standard protocols. Qualified sequences with 97% similarity threshold were assigned to the same operational taxonomic units (OTUs) (Bi et al., 2022). The BLAST tool classified all OTU representative sequences into different taxonomic groups against Silva138 Database. QIIME software (v1.8.0) calculated richness and diversity indices. Principal coordinates analysis (PCoA) and heatmap figures were performed by R (v3.6.0) based on the Bray Curtis algorithms distance. The Metastats by mothur program (v1.34.4) compared treatment differences in bacterial communities. Functional prediction of the microbial communities was based on

FAPROTAX. Python software (v2.7) conducted LEfSe (linear discriminant analysis (LDA) Effect Size) analysis.

2.7 Data analysis

All data was collated using Microsoft Excel 2019. Means and standard error values were calculated using SPSS software version 26. The permutation test used ANOSIM to analyze differences at the community level. Fisher's least significant difference test (LSD) and Duncan's test were used for multiple comparisons of significance among different treatments ($P < 0.05$). The two-tailed Student's t test was used to calculate significance between samples. Figures were generated using Origin (v2022) and R (v4.3.1). The non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances was performed to visualize microbial community composition at the phylum level. To reveal the influential factors of the bacterial relative abundance, we applied Mantel tests to evaluate the correlations between bacterial relative abundance and bio-abiotic drivers. Variation partitioning analysis (VPA) was also used to assess the effect of soil and microbial properties on bacterial relative abundance.

3. Results

3.1 Plants agronomic traits and photosynthetic characteristics

Plant height was significantly impacted by light intensities (Fig. 2a,i). Under moderate and high light intensity, plant height was higher than that under low light. However, there was no significant change in plant height among different seasons. The leaf area of *N. benthamiana* decreased with the planting seasons under low and moderate light intensity, while it showed no significant differences among seasons.

The dry weight of shoots was negatively influenced by planting seasons under all light intensity in our study (Fig. c). However, the degree of decline under different light intensities was different. Under low light intensity, the average dry weight of shoot at 2nd and 3rd season was 20% and 50% lower than the dry weight of shoot at the 1st season, respectively. And the difference in dry weight of shoots among seasons under low light was not significant. Under moderate light intensity, the average dry weight of shoot at 2nd and 3rd season was 25% and 64% lower than the dry weight of shoot at the 1st season, respectively. Under high light intensity, the average dry weight of shoot at 2nd and 3rd season was 39% lower than the dry weight of shoot at the 1st season.

As shown in Fig. 2d, the root-shoot ratio of *N. benthamiana* under low light intensity was significantly lower than that under moderate and high light intensity. There was no obvious difference in root-shoot ratio between moderate and high light or among different seasons.

Under moderate and high light intensity, the net photosynthetic rate of *N. benthamiana* was significantly higher than that under low light. As Fig. 1e shows, the net photosynthetic rate decreased with planting

season regardless of light intensity. The stomatal conductance was higher under moderate and high light intensity while there was no significant difference between different seasons. At the 1st season, the transient transpiration rate showed a positive correlation with light intensity, and high light intensity induced the greatest transient transpiration rate. However, the transient transpiration rate decreased with planting season under all light intensity, and there was no significant difference among different light intensities at the 3rd season. The intercellular CO_2 concentration decreased with planting season under low and moderate light intensities, while there were no apparent changes among seasons under high light.

3.2 Plant antioxidant enzymes activity and hormones contents

As shown in Fig. 3a, the activity of SOD under low light intensity did not differ among different seasons. However, under moderate and high light intensities, the activity of SOD was significantly high at the 1st season than that at the 2nd and 3rd season.

The activity of POD showed negative correlation with the planting season. The activity of CAT was significantly higher under high light intensity than under low and moderate light, while it did not change significantly among different seasons.

Cytokinin, and gibberellin content were both significant influenced by different light intensity ($P < 0.05$) (Fig. 3e, f). In general, auxin, cytokinin and gibberellin content were higher under low light intensity than under moderate and high light, while the abscisic acid content showed positive correlation with light intensity.

The auxin content of *N. benthamiana* was lower than that at 1st season under low light intensity, while the situation was different under moderate and high light. The average auxin content in the 3rd season under low light was 18% lower than that in the 1st season, while it was 8% and 20% higher than that in the 1st season under moderate and high light intensity, respectively. Changes in the cytokinin content were similar to that in the auxin content. Gibberellin and abscisic acid content were higher at the 3rd season than 1st season. The average gibberellin content at the 3rd season under low□moderate and high light intensity was 20%, 8% and 23% higher than that at the 1st season, respectively. The average abscisic acid content at the 3rd season under low□moderate and high light intensity was 20%, 8% and 23% higher than that at the 1st season, respectively.

3.3 Soil physicochemical properties and allelochemicals

Soil EC showed no significant change under all treatments, while soil pH decreased after *N. benthamiana* was planted (Fig. 4a,b). The decrease in soil pH under moderate and high light was more severe than that under low light. The soil available N and P showed negative correlations with planting season regardless of the light intensities (Fig. 4c,d). The results of GC-MS analysis showed that the soil slurries contained BTEX (benzene, toluene, ethylbenzene, m,p-xylene and o-xylene), ketones, phenol, PAHs (polycyclic aromatic hydrocarbons) and other allelochemical compounds (Fig. 4e). Among them, 2,

4-ditert-butyl phenol, isophorone, azulene and tetramethylbenzene was the most abundant compounds. 2, 4-ditert-butyl phenol was known as the allelochemical that was relatively high in content and has been shown to be harmful to plant growth. The relative content of 2, 4-ditert-butyl phenol in legacy soils was higher than that in the initial soil. As the light intensity increased, the content of 2, 4-ditert-butyl phenol also increased, under medium and high light intensities, 2, 4-ditert-butyl phenol became the substance with the highest relative content.

3.4 Soil bacterial community composition and function

As shown in Fig. 5a, Proteobacteria was the dominant specie in all soils, after *N. benthamiana* was planted, the relative abundance of Proteobacteria in the legacy soil increased compared to the initial soil. Compared to the initial soil, the relative abundances of Bacteroidetes, Verrucomicrobia, Spirochaetes and Planctomycetes in legacy soil were increased. The relative abundance of Bacteroidetes in initial soil was 5.98%, and in legacy soils it was two or three times bigger. The relative abundances of Verrucomicrobia, Spirochaetes and Planctomycetes in initial soil were 0.33%, 0.02% and 0.06%, respectively. However, after *N. benthamiana* was planted, the relative abundances of these three kinds of bacteria were significantly increased by dozens of times. The relative abundances of Actinobacteria, Chloroflex and Firmicutes decreased with planting season. For example, in initial soil, the relative abundance of Actinobacteria was 17.07%, when the first season of *N. benthamiana* was harvested, the relative abundance of Actinobacteria in legacy soils were around 8.00% (no significant differences between three light intensities), and when the third season of *N. benthamiana* was harvested, the relative abundance of Actinobacteria in legacy soils decreased to ~ 4.00%.

According to the results of NMDS (Fig. 5b), the soil microbial community was significantly changed and was more affected by the continuous season. The initial soil and the third quarter soil microbial communities were significantly different from the soil microorganisms in the first and third quarters. Figure 5c shows the results of alpha diversity analysis of soil bacterial community. The number of observed species and chao1 index of different soils did not differ significantly. The Shannon indexes of legacy soils were significantly bigger than that of initial soil, except for M_3 treatment. Generally, after *N. benthamiana* was planted, the bacterial community diversity of legacy soil increased. The goods_coverage of each soil was higher than 98%, indicating the results can truly reflect the bacterial community in each soil samples. As for the bacterial function, significant changes occurred after continuous cropping, such as significant decrease in chemoheterotrophy, fermentation, nitrogen fixation and animal parasites or symbionts with the increase of continuous cropping seasons. In contrast, predatory or exoparasitic, nitrogen respiration, denitrification and phototrophy were significantly up-regulated (Fig. 5d).

According the PERMANOVA analysis (Fig. 6a,b), continuous cropping season had a significant impact on soil microbial composition (R^2 = 0.367, F = 9.266, P = 0.001), while light intensity had no significant impact on soil microbial community structure (R^2 = 0.100, F = 0.829, P = 0.590). When continuous cropping seasons were analyzed separately (Fig. 6c,d), the effect of light intensity on soil microbial

structure in the first season (R^2 = 0.247, F = 0.986, P = 0.452), while with the increase of continuous cropping, the effect light intensity on soil microbial community structure became significant in the third season (R^2 = 0.321, F = 1.421, P = 0.027).

Linear discriminant analysis (LDA) effect size (LEfSe) was used to discover the differential microbes (biomarkers) and estimate the biomarkers' effect size. There were significant differences between the three light treatments in the third season, indicating that the soil microbial community structure was significantly changed after three seasons of crop planting (Fig. 6e,f,g). Compared to the initial soil, low light treatment significantly enriched c_Verrucomicrobiae, f_Alphaproteobacteria, s_*Bacterium_Ellin518*, f_Rhizobiales, g_*Pseudolabrys* and g_*Micropesis*; moderate light treatment significantly enriched c_Alphaproteobacteria, o_Micropepsaceae, g_*Dongia* and g_*Occallatibacter*, and high light treatment significantly enriched c_Alphaproteobacteria, o_Micropepsaceae, g_*Occallatibacter*, f_Rhizobiales, g_*Solibacter*, c_Deltaproteobacteria, g_*Dongia* and g_*Pseudolabrys*. There was no biomarkers among different light treatments in the first season, however, in the third season, different biomarkers were found from different light treatments (Fig. 6h). Light-induced different soil legacy under three seasons of different light intensity conditions, the microorganisms significantly enriched in low light treatment were *s_bacterium_Ellin518*, *g_Arachidicoccus* and *g_Enterococcus*; the microorganism significantly enriched in medium light treatment was f_Chitinophagaceae; while the microorganisms significantly enriched in high light treatment were: f_unidentified_Alphaproteobacteria, *g__unidentified_Hyphomonadaceae* and *s_Woodsholea_maritima*.

3.5 Correlations between soil properties, allelochemicals and bacterial communities

The Pearson's correlation analysis was conducted between soil properties and soil bacterial community composition and function (Fig. 7a,b). Except soil EC and BD, all soil properties showed significant correlation with most taxa and function. Except for those with less abundances, such as thioethers, aldehydes, alkanes, and alcohols, all allelochemicals showed significant correlation with most taxa and function. The correlation results of differential allelochemicals and core microbiomes showed that *unidentified_Burkholderiaceae*, *Streptomyces*, *Massilia*, and Bordetella were significantly negatively related with most allelochemicals ($P < 0.05$), while Occallatibacter, Candidatus_Solibacter, Dongia, and Bauldia were significantly negatively related with most allelochemicals ($P < 0.05$). Mantel test analysis confirmed the above results, finding that soil bacterial community composition and function was significantly correlated with AN, AP, SOC, and BTEX, phenol, ketones, and butanoic acid (Fig. 7c). However, soil bacterial community diversity was not affected by soil properties and allelochemicals. Variation partitioning analysis (VPA) was conducted to address the relative role of soil properties and allelochemicals and their combined effect on microbial composition and function (Fig. 7d,e). The results revealed a higher effect of allelochemicals than that of soil properties for both microbial composition and microbial function. For microbial composition, the individual explanation rate for allelochemicals and soil properties were 0.2708 and 0.0255, respectively; and their shared explanation rate was 0.7037.

As for microbial function, the individual explanation rate for allelochemicals and soil properties were 0.3065 and 0.0222, respectively; and their shared explanation rate was 0.6712.

3.6 Effects of legacy soil slurry

As shown in Fig. 8, legacy soil slurry notably decreased *N. benthamiana* productivity under all light intensities compared to the control group. However, after the slurry of legacy soil was filtered, the negative effects were partly offset, and an increase in *N. benthamiana* dry weight of shoot was found. Allelochemicals in legacy soil slurry were also measured (Fig. 8b). All these three allelochemicals, BTEX, phenol and PAHs were significantly decreased after filtered 0.22um PTFE membrane ($P < 0.05$).

Heatmap illustrating the differential microbes in different treatments (Fig. S2a). The abundance profiles of microbes were divided into six groups. Specifically, Group I, II and IV microbes were significantly up-regulated under M-F0.22 and M-CK treatments, while Group III, V and VI microbes under L-UF and M-UF treatments. A more detailed differential analysis was conducted to reveal the certain genus contributed to plant growth (Fig. 9a,b). The microorganisms significantly enriched in M-F0.22 treatment were: *Flavobacterium*, *Luteimonas*, *Rhodanobacter*, *Gaulobacter*, and *Kribbella*; The microorganisms significantly enriched in the M-CK treatment were: *Flavobacterium* and *Pseudomonas*; Compared with M-F0.22 and M-CK treatments, the microorganisms significantly enriched in the M-UF treatment were: *Bauldia*, *Sphingomonas*, *Rhodoplanes*, *Candidatus_Solibacter*, *Dokdonella, SM1A02*, *SH-PL 4*, and *Leptothrix*. The indicator analysis confirmed that *Rhodanobacter*, *Pseudomonas* and *Flavobacterium* were significantly enriched in M-F0.22 and M-CK treatments; While *Dongia*, *Bauldia*, and *Dokdonella* were significantly enriched in L-UF and M-UF treatments (Fig. 9c).

4. Discussion

Here, we characterize the light-induced root allelochemicals and microbiomes shifts in *N. benthamiana*. The microbial community regulated by allelochemicals may produce different responses to continuous cropping under different light conditions. Under moderate light intensity conditions, the accumulation of allelochemicals, especially 2, 4-ditert-butyl phenol and dimethylbenzene, leads to the imbalance of microbial community and function, with less beneficial microbes (*Streptomyces*, *Massilia*) and less nitrogen fixation function, and thus inhibits plant growth and aggravates the obstacle of continuous cropping. In contrast, under high light intensity conditions, lower doses of 2, 4-ditert-butyl phenol stimulate the generation of plant hormones directly or indirectly through the recruitment of beneficial microorganisms, such as *Pseudomonas* and *Flavobacterium*, thus promoting plant growth to alleviate the CCO.

4.1 Imbalanced microbes caused decrease in plant yield under continuous cropping

Continuous cropping usually causes decrease in plant yield and degradation of soil quality. In our study, the biomass of *N. benthamiana* decreased with planting seasons. Under all light conditions, the dry

weight of shoot and leaf area at the 3rd season were both lower than at the 1st season, the net photosynthetic rate and transpiration rate also decreased significantly with the continuous cropping season, suggesting that continuous cropping affects plant growth and yield by inhibiting photosynthesis. The investigation of plant protective enzyme activity showed that the legacy soil of different light intensities influenced SOD activity differently. Under low light intensity, SOD activity did not differ among seasons. However, under moderate and high light intensity, SOD activity decreased significantly at the 2nd season, indicating the legacy soil caused damage on plant protective enzyme system. SOD is a main protective enzyme of plant, it is responsible for the direct reactive oxygen species detoxification in plant tissues (Vuleta et al., 2016). In our study, the dry weight of shoot showed similar changes with SOD activity. It seems that the legacy soil under moderate and high light reduced *N. benthamiana* SOD activity and decreased photosynthetic rate, leading to significantly reduction in plant biomass. These results were consistent with the findings of the previous studies (Chi et al., 2013; Niu et al., 2017; Zhang et al., 2022; Ma et al., 2023).

Soil properties also changed with planting season in our study. Soil pH and soil available N/P decreased with planting season under all light intensities. Under continuous cropping conditions, soil acidification is widespread, mainly due to the accumulation of phenolic acids secreted by the roots. Lower pH will not only affect the content of available nitrogen and phosphorus, but also inhibit the absorption of nitrogen, phosphorus, potassium and other nutrients by roots, and further affect plant growth (Chen et al., 2023). In addition, allelochemicals were also significantly altered under continuous cropping conditions, which in this study caused the accumulation of phenol (e. g. 2,4-ditert-butyl phenol) and BTEX (e. g. dimethylbenzene). These allelochemicals can have both beneficial and detrimental effects on plants, which is usually concentration-dependent. They may promote plant growth at low concentrations, but inhibit root development and cause self-toxicity at high concentrations (Calabrese et al., 2007).

Root exudates not only serve as a medium of communication between plants and soil microorganisms, but also affect plant growth and health by changing soil physicochemical properties and microbial community structure. In this research, continuous cropping distinctly altered the allelochemicals and then influenced soil bacterial community composition and function, which may be an important reason for CCO. Our results revealed that the relative abundance of beneficial taxa, such as *Streptomyces* and *Massilia*, significantly decreased with the *N. benthamiana* growth seasons. Both these two genera bacteria promote plant growth. *Massilia* is a class of Gram-negative bacteria widely found in plant inter-roots that promotes nitrogen uptake and utilization (He et al., 2024), and *Massilia sp. Pn2* strains have the ability to efficiently degrade polycyclic aromatic hydrocarbons (PAHs), e.g., more than 95% of phenanthrene degradation within 3 days (Liu et al., 2014). It significantly reduced pollutant accumulation in plants by colonizing the root surface, while promoting root development and biomass increase. In addition, *Massilia* enhances plant stress tolerance by regulating the synthesis of plant hormones (e.g., auxin and cytokinins) under saline or drought stress. Its secreted metabolites can activate the plant antioxidant system and reduce cell damage by reactive oxygen species (ROS) (He et al., 2024). *Streptomyces* is a class of Gram-positive bacteria, which are capable of producing a variety of antibiotics (e.g., streptomycin, actinomycetes) that directly inhibit the growth of soil pathogens (e.g., *Fusarium*,

Pythium) and reduce the incidence of soil-borne diseases (Tian et al., 2023). In addition, *Streptomyces* can activate soil nutrients such as insoluble phosphorus and potassium through the secretion of organic acids and phosphatases, thus improving soil fertility (Nazari et al., 2023). In this study, we demonstrated that during continuous cropping of *N. benthamiana*, the "suppressing beneficial microbes" substances (e.g. 2, 4-ditert-butyl phenol) in root exudates disturbed the rhizosphere microorganism community and inhibited the abundance or function of beneficial microbial taxa.

In summary, plant-soil-microbe interactions play a key role in the development of continuous crop obstacles. The mechanisms of CCO of N. benthamiana in this study is the accumulation of negative soil legacy, i.e., the accumulation of allelochemicals (2, 4-ditert-butyl phenol) secreted by the root alters the structure and function of the microbial community, reduces the abundance of beneficial taxa (Streptomyces, Massilia) and nitrogen fixation function, and leads to the deterioration of the soil environment, which then inhibits photosynthesis and affects the growth of the subsequent crops.

4.2 High-light induced soil legacy alleviates the negative effects of continuous cropping

Legacy soils, influenced by varying light intensities under three seasons of continuous cropping, exert detrimental effects on plant growth and productivity. Notably, high light conditions appear to mitigate these adverse impacts associated with continuous cropping. Specifically, plant dry biomass exhibited a marked decline over time under moderate light intensity, whereas the differences in plant dry biomass across seasons were not statistically significant under low light intensity. In contrast, under high light intensity, a significant reduction in plant dry biomass was observed during the second season; however, no further decrease was detected in the third season compared to the second. Results from GC-MS analysis indicated that the relative content of 2,4-ditert-butyl phenol increased with light intensity. Additionally, certain beneficial bacteria were found to be enriched under high light intensity treatment. This suggests that under such conditions, legacy soils accumulate greater quantities of 2,4-ditert-butyl phenol, which may trigger a "cry for help" strategy, enabling plants to recruit beneficial microbes that enhance their resilience to environmental stresses. Previous research has demonstrated that triterpenoid compounds found in melon can regulate the rhizosphere microbiome and bolster plant disease resistance (Zhong et al., 2022). Furthermore, in addition to its inhibitory effects on plant growth, 2,4-ditert-butyl phenol exhibits biological preventative properties, capable of inhibiting the growth of pathogenic fungi in the plant rhizosphere, thereby alleviating the obstacles associated with continuous cropping of *N. benthamiana*.

High light stress induces plants to recruit beneficial rhizosphere microorganisms, thereby alleviating the challenges associated with continuous cropping. Under conditions of light stress, these microorganisms can provide both direct and indirect support to enhance plant stress resistance. Similar to their protective roles under salt or water stress (Fadiji et al., 2023), some researchers have proposed that rhizosphere microorganisms possess the ability to regulate plant hormone synthesis, including abscisic acid (ABA) and various secondary metabolites (e.g., antioxidants), to assist plants in coping with light

stress events (Ren et al., 2019). Previous studies have reported that light stress-induced microorganisms can promote plant growth in the presence of pathogens (Liu et al., 2024; Zhao et al., 2023). Our findings indicate that high light stress enriches beneficial microorganisms, leading to enhanced stress resistance. Specifically, high light stress fosters a more effective consortium of microorganisms capable of degrading autotoxins compared to moderate light stress. Under high light conditions, notable enrichments of *Alphaproteobacteria* and *Hyphomonadaceae* were observed, while *Chitinophagaceae* was predominantly enriched under moderate light conditions. Conversely, *Verrucomicrobiae*, *Arachidicoccus*, and *Enterococcus* were primarily enriched under low light stress. This suggests that plants, in response to varying light conditions, indeed recruit different rhizosphere microorganisms.

Signals arising from aboveground light stress are integrated by beneficial belowground microbes, prompting plant responses to both biotic and abiotic stresses. The perception of these signals by plant leaves modifies root secretion patterns, enabling rhizosphere microorganisms to assist in stress mitigation. Previous studies have demonstrated that signals from foliar pathogens or insect damage also alter root exudates, thereby triggering beneficial belowground microbes to enhance plant stress tolerance (Yuan et al., 2016; Luo et al., 2022; Hou et al., 2021). Fang et al. (2024) employed a combination of metabolomics and microbiome analysis to investigate the effects of various light stress conditions on the growth of *Panax pseudoginseng*. Their study found that high light conditions facilitated the recruitment of beneficial soil microorganisms by altering metabolic pathways and root exudate profiles. High light stress enhances metabolic activity, resulting in increased exudation of various flavonoids, which subsequently enriches the beneficial microbiome while suppressing soil-borne pathogens. In the present study, in addition to phenolic acids, significant associations were identified between soil microorganisms and other compounds such as PAHs, BETX, and ketones. This suggests that these compounds may also play a role in microbial reorganization under light stress treatments. Thus, high-intensity light stress promotes secondary metabolism, which inhibits detrimental microorganisms and fosters beneficial ones, ultimately alleviating the obstacles posed by continuous cropping.

Overall, high light stress alleviates the challenges associated with continuous cropping by modifying the composition and function of rhizosphere microorganisms through alterations in the allelochemicals secreted by the root, which can either inhibit or promote microbial growth. However, this study did not measure the transcriptome or metabolome, nor did it include subsequent validation of pure bacterial strains. Consequently, the potential mechanisms by which aboveground light stress signaling influences root secretion and recruits rhizosphere microorganisms warrant further investigation, given the complexity of light signaling in regulating plant growth and metabolism. In the future, multi-omics technologies such as transcriptome, metabolome, and metagenome can be used to comprehensively analyze the plant-soil-microbiome crosstalk so as to provide theoretical basis and technical support for the prevention and control of *N. benthamiana* CCO.

Conclusion

The release of *N. benthamiana* allelochemicals and its regulation of the rhizosphere microbial community composition and function under different light conditions are the main driver affecting the differential response of *N. benthamiana* to continuous cropping. Under moderate light intensity conditions, the accumulation of allelochemicals, especially 2, 4-ditert-butyl phenol and dimethylbenzene, leads to the imbalance of microbial community and function, with less beneficial microbes (*Streptomyces*, *Massilia*) and less nitrogen fixation function, and thus inhibits plant growth and aggravates the obstacle of continuous cropping; while under high light intensity conditions, lower doses of 2, 4-ditert-butyl phenol stimulate the generation of plant hormones directly or indirectly through the recruitment of beneficial microorganisms, such as *Pseudomonas* and *Flavobacterium*, thus promoting plant growth to alleviate the CCO. These findings can provide new strategies from the perspective of plant-microbe interactions for the prevention and control of CCO and promote the soil sustainability.

Declarations

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All authors contributed to the study conception and design, and final paper writing. Material preparation, data collection and analysis were performed by Xiao Tan, Zhihui Wang, Songsong Li, and Bo Tan. The laboratory operation and equipment maintenance were conducted by Wenhua Zhuang and Liu Chao. The first draft of the manuscript was written by Xiao Tan, Zhihui Wang, Songsong Li, and Yuxin He and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

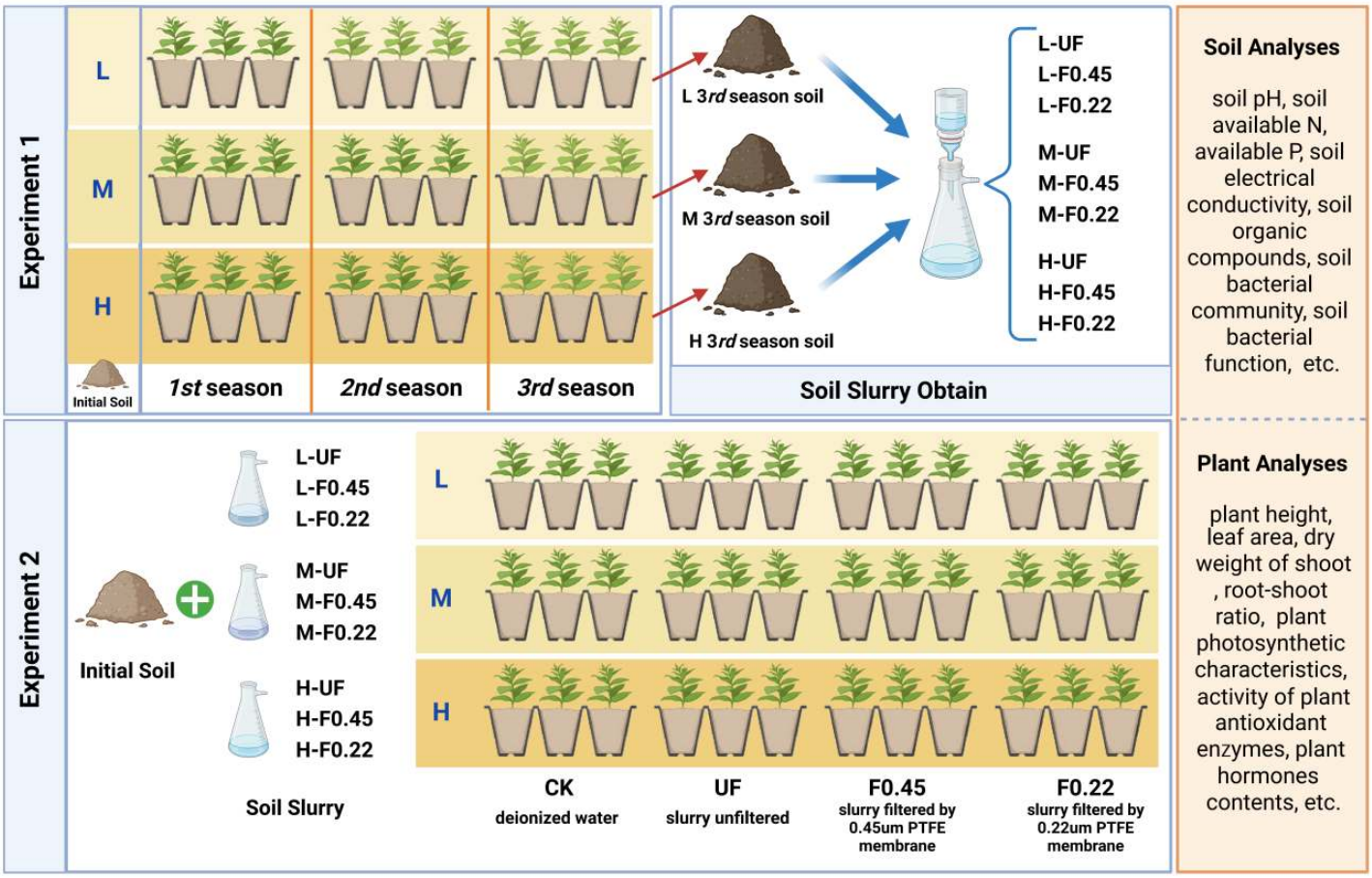

Figure 1

Flow diagram of the experimental design. In experiment 1, we grew N. benthamiana under three different light intensities for three continuous seasons to test the responses of plant, soil properties and rhizosphere microbes to the light and continuous cropping; After experiment 1, for each light-conditioned legacy soil, the soil slurry was collected and divided into three parts for experiment 2, one part was filtered through 0.45um filter polytetrafluoroethylene (PTFE) membrane, one part filtered through 0.22um PTFE membrane, and no membrane filter for the last part. Unfiltered soil slurry (UF), 0.45um-filtered (F0.45), 0.22um- filtered (F0.22) and deionized water (CK) was added to a pot of initial soil (soil that never been planted before), and then N. benthamiana was grew to further confirm that the negative soil-legacy (self-toxic exudates and microbiomes) was the main cause affecting the growth of subsequent plants.

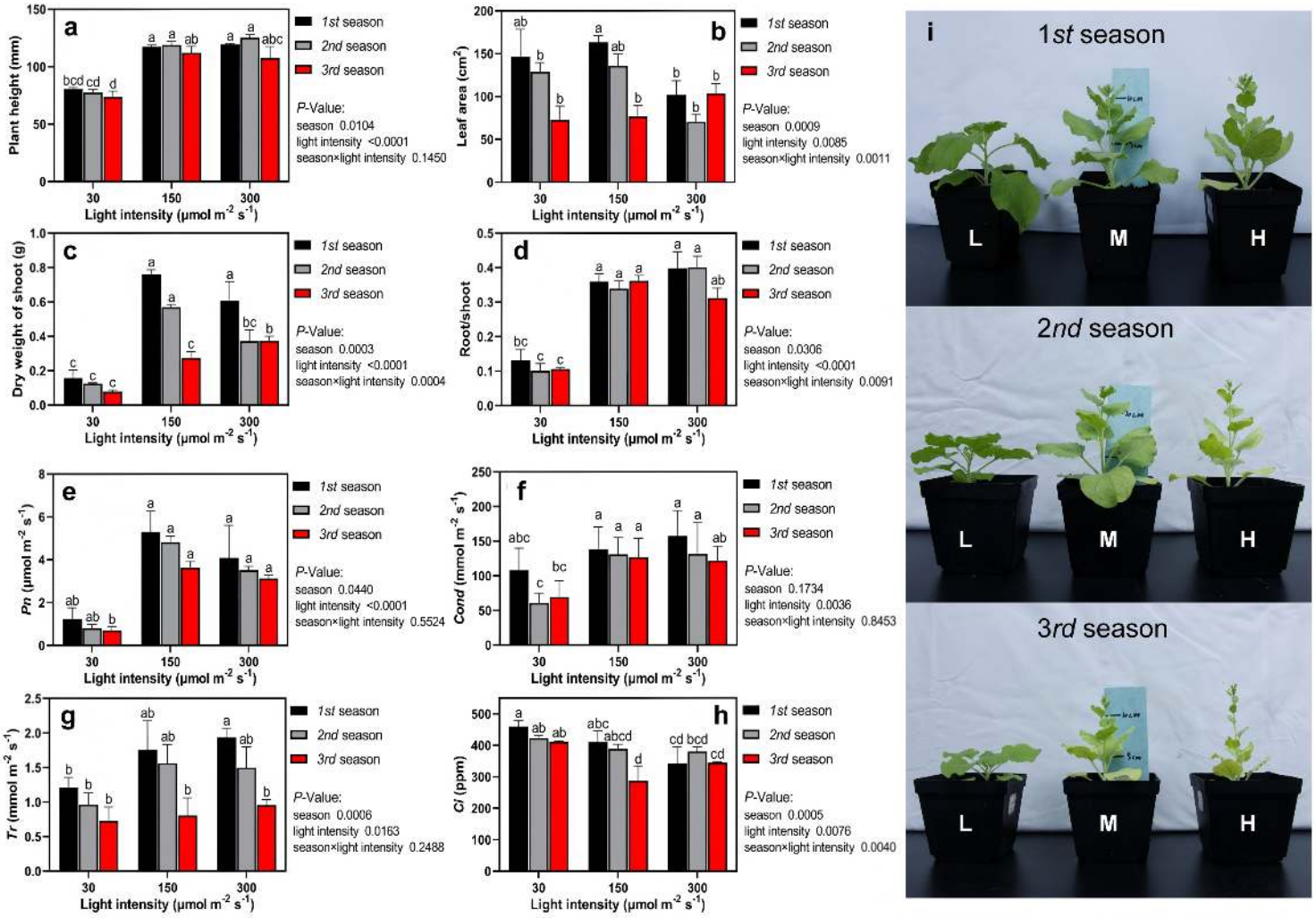

Figure 2

Plant agronomic traits (a,b,c,d), photosynthetic characteristics (e,f,g,h) and growth visualization (i) of *N. benthamiana* under different treatments. Error bars represent the standard error of the means (n=3). Bars with different lowercase letters are significantly different according to the Duncan test at $P < 0.05$.

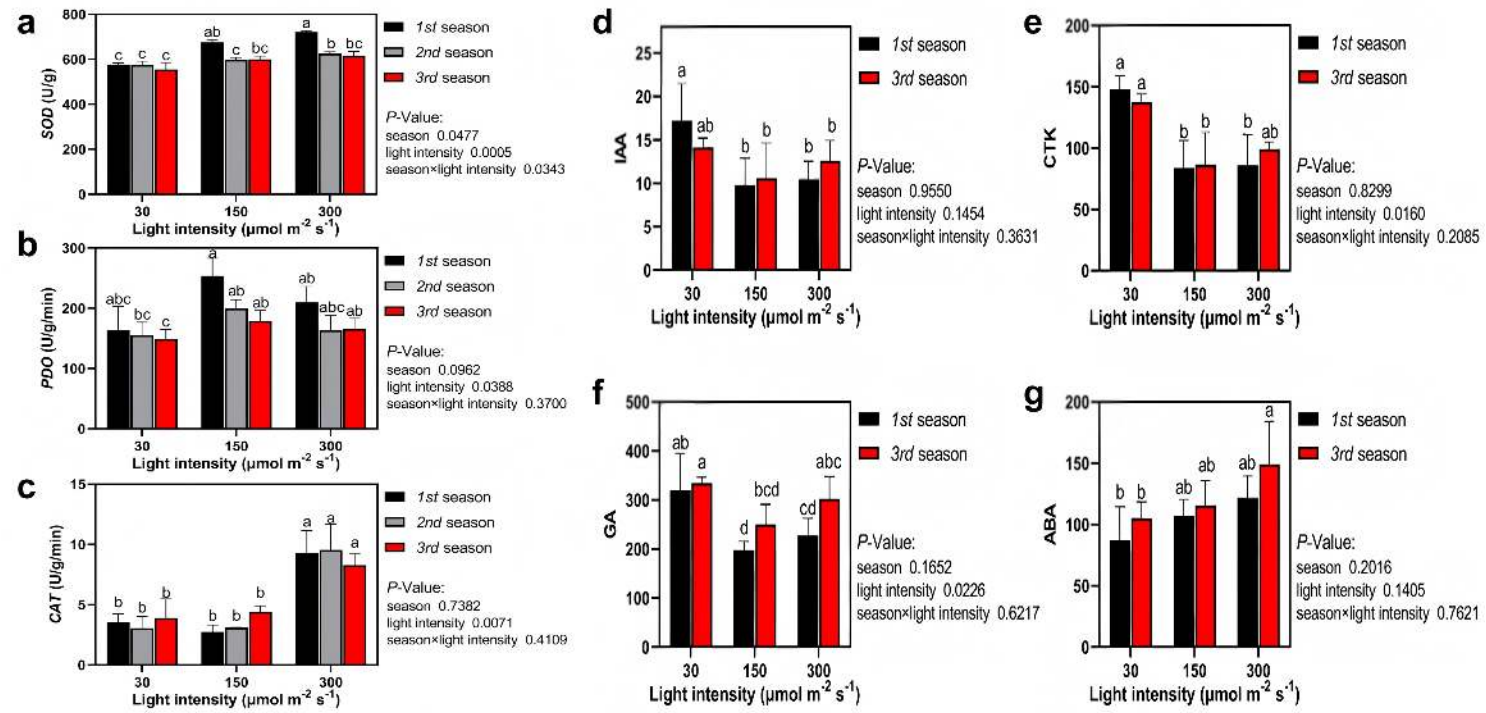

Figure 3

The activity of antioxidant enzymes (a,b,c) and hormones contents (d,e,f,g) of *N. benthamiana* under different treatments. Error bars represent the standard error of the means (n=3). Bars with different lowercase letters are significantly different according to the Duncan test at P < 0.05.

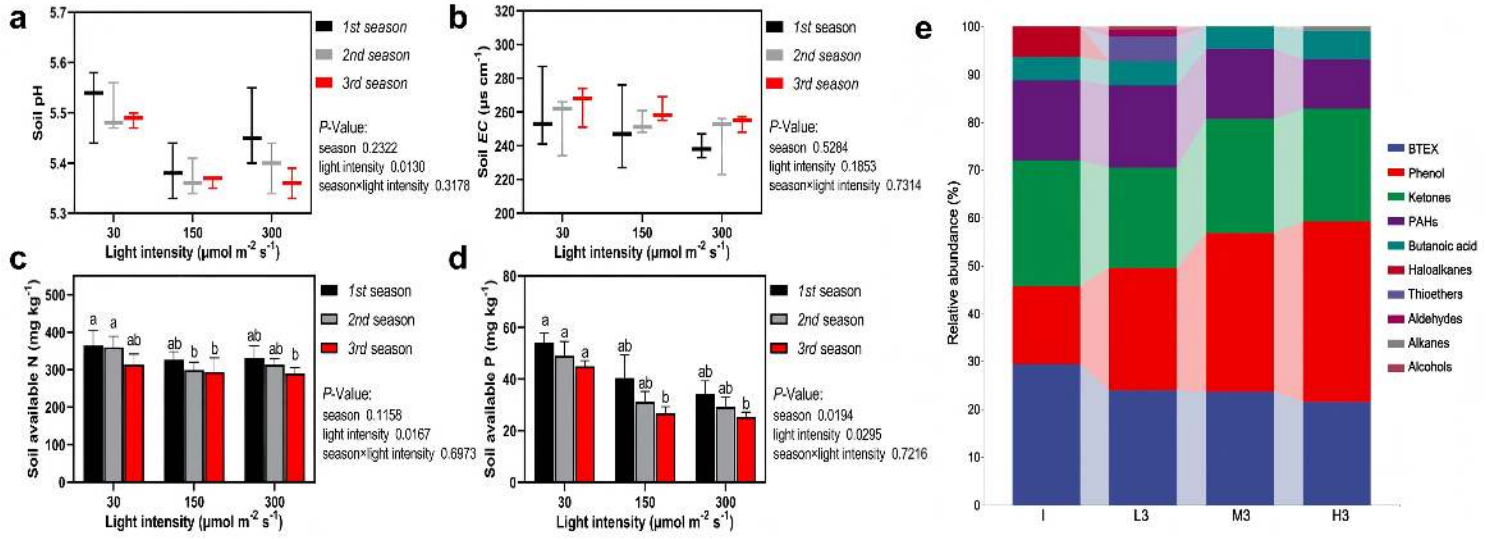

Figure 4

Soil pH (a), electrical conductivity (b), available nitrogen (c) and available phosphorus (d) under different treatments. Error bars represent the standard error of the means (n=3). Bars with different lowercase letters are significantly different according to the Duncan test at P < 0.05. Relative content of soil organic

compounds (e) in initial soil (I), legacy soil in which *N. benthamiana* has been planted for three seasons under low (L3), medium (M3) and high (H3) light.

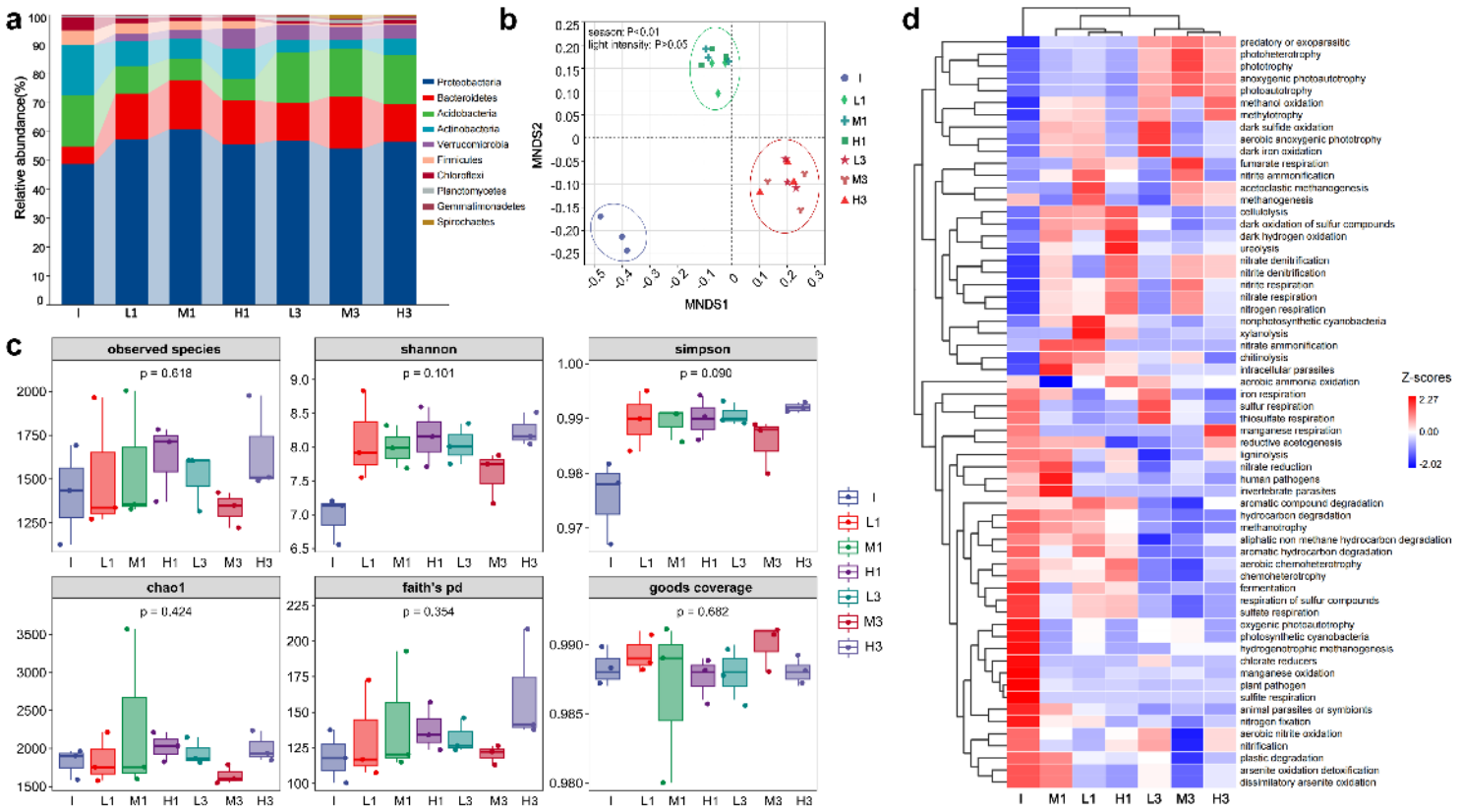

Figure 5

Bacterial relative abundance at phylum level (a), community diversity (b, c) and function (d) in initial soil (I), legacy soils in which *N. benthamiana* has been planted for one season under low (L1), medium (M1) and high (H1) light, legacy soils in which *N. benthamiana* has been planted for three seasons under low (L3), medium (M3) and high (H3) light.

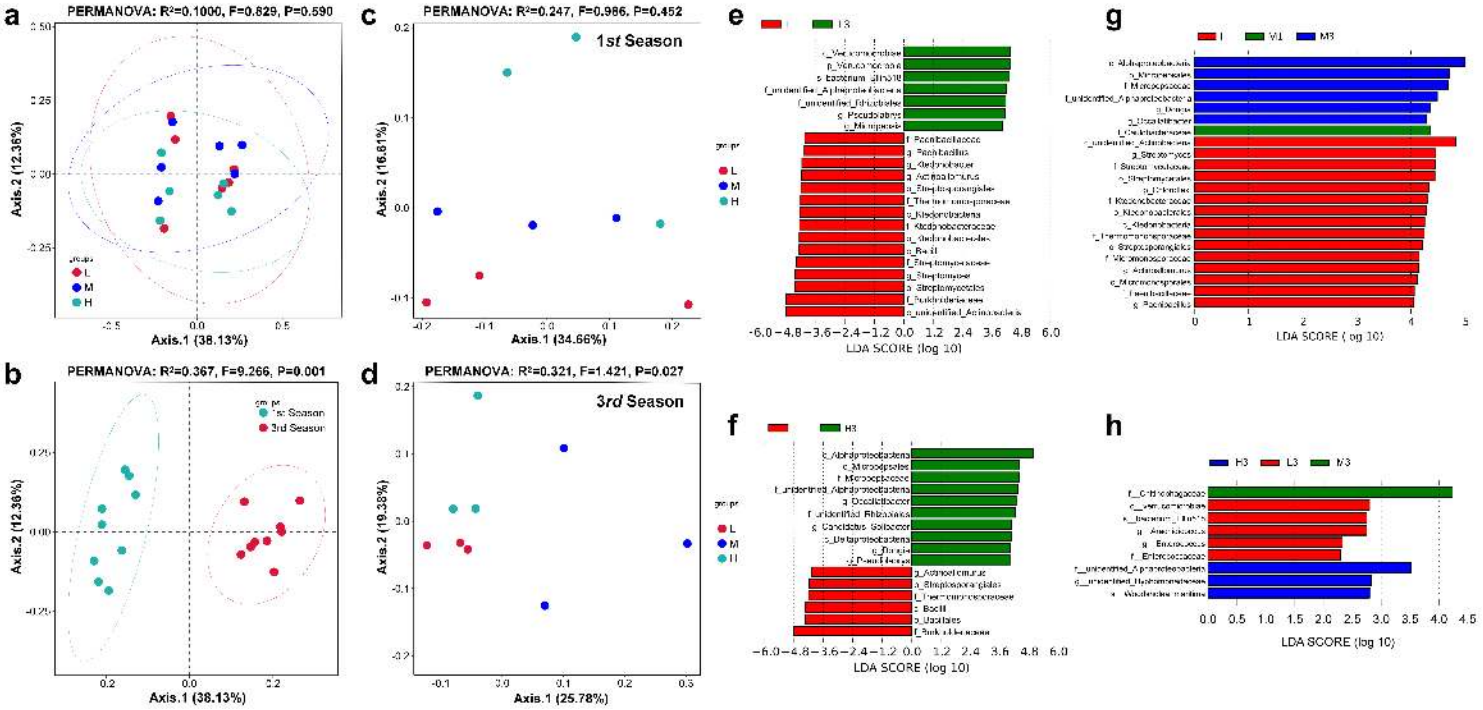

Figure 6

PCoA ordination of the Bray-Curtis dissimilarity matrix (OTU level) for all treatments with light and season groups (ab) and for 1st and 3rd season separately (cd), statistical analysis was performed using PERMANOVA; Linear discriminant analysis effect size (LEfSe) results in the three continuous season under different light intensity treatments (efgh). The taxa with absolute LDA ≥3.5 and P < 0.05 is shown.

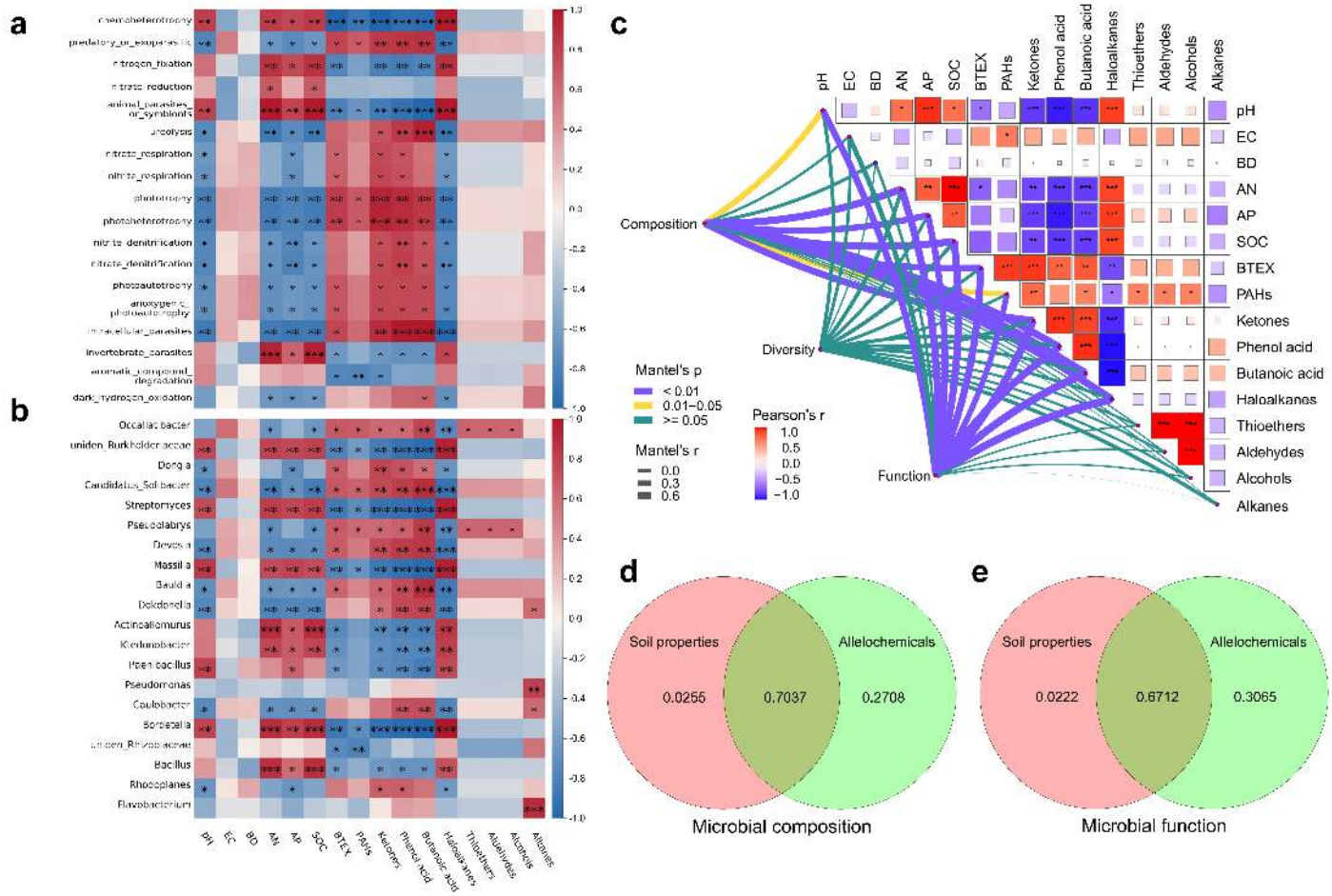

Figure 7

Correlation heatmap of soil properties, allelochemicals and core function and core microbes (ab); Mantel test of soil properties, allelochemicals and microbial composition/diversity/function (c); VPA analysis of microbial composition and function (de) with soil properties and allelochemicals. *, ** and *** indicate significant at P<0.05, P<0.01 and P<0.001, respectively.

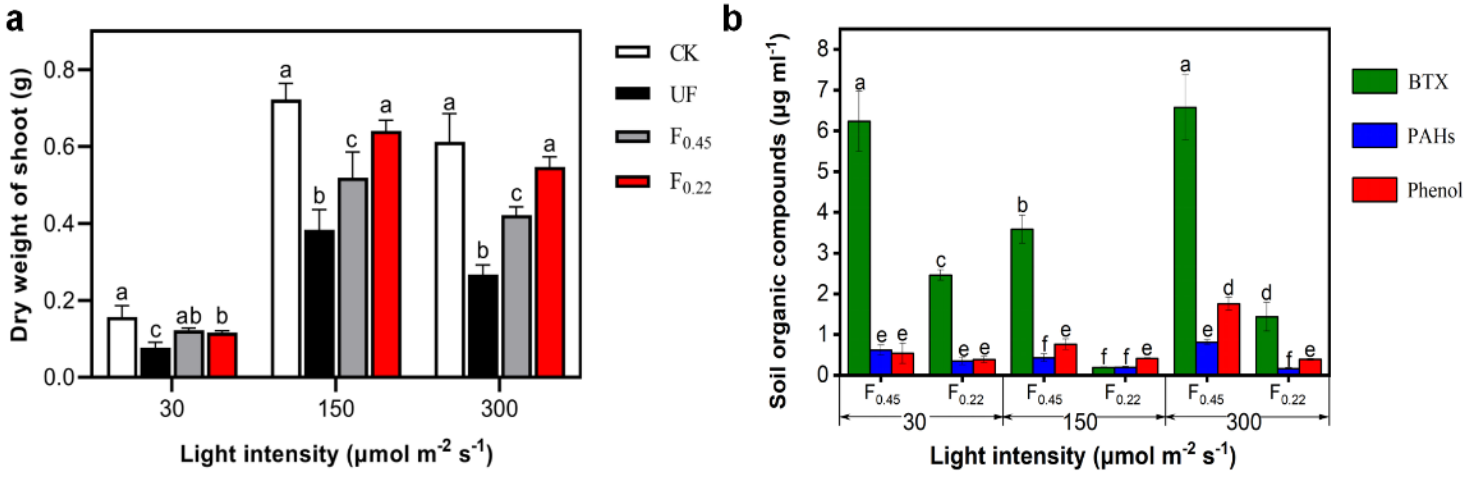

Figure 8

The dry weight of shoot of N. benthamiana growing in initial soils plus deionized water (CK), unfiltered legacy soil slurry (UF), slurry filtered by 0.45um PTFE membrane (F0.45), and slurry filtered 0.22um PTFE membrane (F0.22). Error bars represent the standard error of the means (n=3). Bars with different lowercase letters are significantly different according to the Duncan test at P < 0.05, and the significant difference was only compared under the same light intensity.

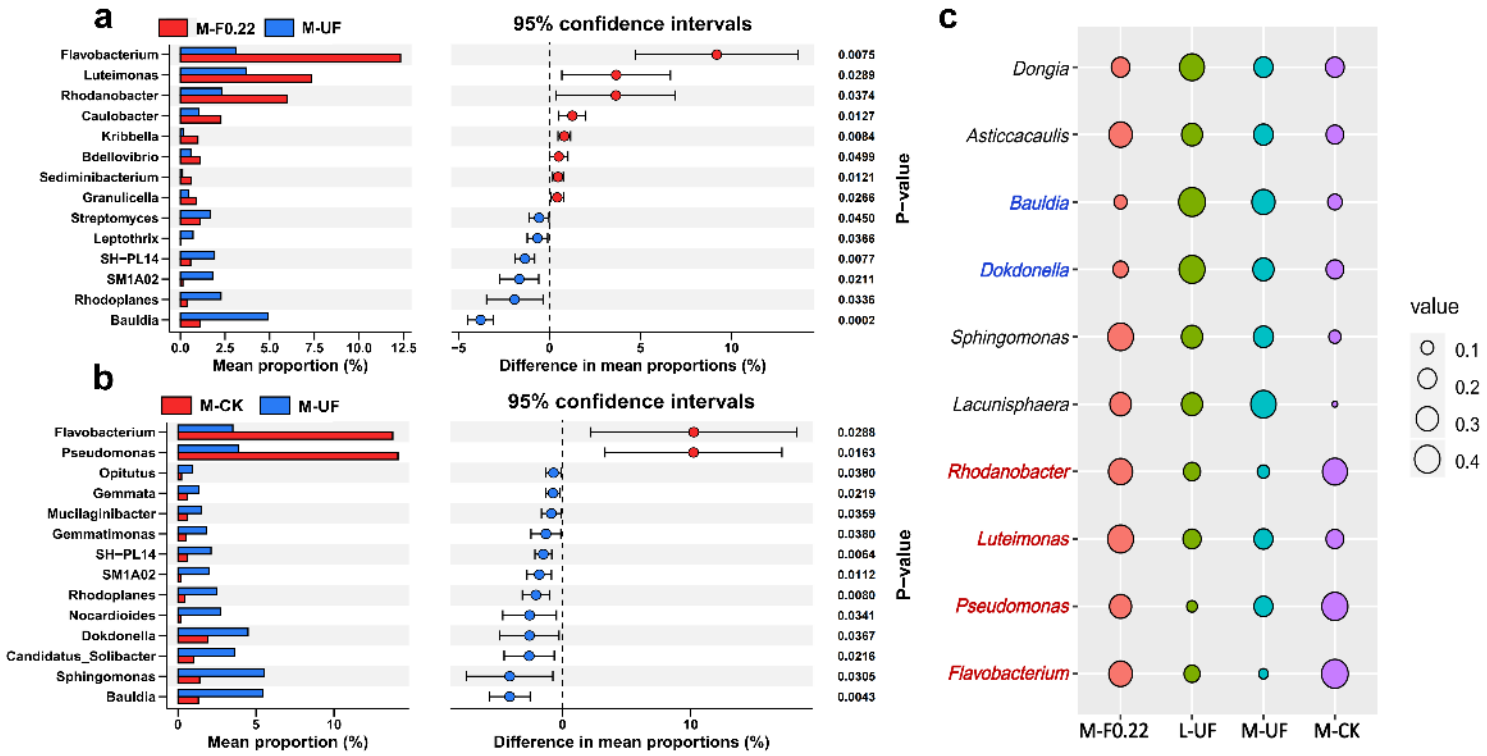

Figure 9

Differentially abundant genera identified by STAMP analysis between M-F0.22, M-CK and M-UF (ab); bubble chart of the genus microbes with significant difference in soil slurry experiment (c). L-UF and M-UF represent unfiltered legacy soil slurry in which *N. benthamiana* has been planted for three seasons under low, and medium light, respectively; M-F0.22 represents legacy soil slurry filtered 0.22um PTFE membrane in which *N. benthamiana* has been planted for three seasons under medium light; M-CK represents the pot with deionized water.