

Soil metabolic factors, not pathogens, drive replant disease: insights from a 20-year soil legacy experiment

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Abstract

Background

Replant diseases (RDs) often stem from nutrient deficiency, allelopathy, or pathogen accumulation. However, the RDs of certain crops are long-lasting and their causes remain unknown. We examined *Panax quinquefolius* RD in a space-for-time soil sequence representing crop rotation restoration over 1, 10, and 20 years using multiomics and bioassays.

Results

Compared with the soils with no ginseng cultivation history, we found 110 potential influencing factors (not including pathogens) related to RD in the 1-year rotation soil, 53 of which remained unrestored after 20 years. Soil pH and the levels of organic nutrients (amino acids, carbohydrates, and alditols), allelopathic-promoting metabolites (phenolic acids, amines, pyridines, etc.), and beneficial bacteria (*Sphingomonas*, *Burkholderia-Caballeronia-Paraburkholderia*, and *Terrabacter*) and fungi (*Acremonium*, *Penicillium*, and *Naganishia*) decreased, while the levels of allelopathic inhibitory factors (pyruvic and fatty acids) increased. The overall soil metabolism was downregulated. We confirmed that decreased organic nutrient levels and increased levels of allelochemical inhibitory substances impaired *P. quinquefolius* growth. Network analysis showed that soil metabolic factors rather than microbial factors were dominant.

Conclusions

Through a partial least squares structural equation model, we found that the overall changes in nutrient levels and metabolic and microbial factors contributed to short-term RD (1–10 year) persistence, whereas long-term RD (after 20 years) primarily resulted from disordered soil metabolite levels and pathways. This research will help deepen our understanding of the relationship between RD and potential changes in the factors influencing RD that are caused by soil legacy effects of valuable plants and provide theoretical guidance for effective soil quality improvement.

Background

Replant disease (RD) is a continuous cropping issue in which one crop influences or “conditions” the soil in a specific manner, negatively affecting subsequent crops of the same (or phylogenetically close) plant [1]. RD is a common phenomenon in the cultivation of crops belonging to the Rosaceae, Solanaceae, Cucurbitaceae, and Arecaceae families [1–4]. Notably, RD severely limits sustainable land use and hampers the development of associated industries, leading to significant ecological and economic losses. Nevertheless, the precise causes of RD remain unclear [5].

Plants use various strategies to respond phenotypically to biotic and abiotic factors in their environment, including the absorption of nutrients, secretion of compounds from the roots into the surrounding soil, recruitment of specific microorganisms, and modulation of the soil microbiome [6]. The soil changes induced by these strategies are known as soil “legacies”, and their effect is termed the “legacy effect”[7]. The subsequent processes that regulate or influence growth performance of the same plant, or other plant species through the legacy effect are called “plant–soil feedbacks”[1, 8–11]. Long-term or repeated growth of the same plant can alter soil biotic and abiotic properties and produce stronger legacies, including depletion of specific or preference nutrients, accumulation of toxic metabolites secreted by the plant and their derived metabolites, and buildup of plant species-specific soil pathogens[5, 8, 12]. Therefore, RD can be characterized as an intraspecific negative plant–soil feedback caused by the soil legacy effect [1, 13].

Although inorganic nutrients are important for replant growth, they are not often restricted to managed farmland owing to the addition of artificial chemical fertilizers. Organic nutrients, such as dissolved organic nitrogen (N) [14] and carbon (C) [15, 16], are vital for plant growth, especially in cold regions where plants have a long period of dormancy [14]. Some dissolved organic nutrients are more suitable for absorption and assimilation by specific plants than inorganic nutrients [15, 17], and therefore, these warrant further attention in the context of RD.

The accumulation of metabolites secreted by plants can cause allelopathic autotoxicity [18] and recruit specific microorganisms to shape the soil microbiome, thereby driving plant–soil feedback effects on replant growth and defense [19]. Some studies suggest that allelopathic inhibitory effects can occur in pre-replanted soils with the expected concentrations of accumulated phenolic acids [20–22], whereas other studies indicate that phenolic acids (produced by plants under external stress) exhibit numerous beneficial effects including antifungal and antioxidant effects [23, 24]. Moreover, other metabolites, such as monoamines and polyamines, possess anti-pathogenic and anti-stress properties [25–27] that may alleviate the allelopathic inhibitory effects on crop replant. Furthermore, while the overall soil metabolic process or level is important, this remains an under-researched topic in the context of RD. Hence, investigations into the specific changes that occur in the soil metabolome to increase our understanding of the dynamics of RD are of paramount importance.

Pathogen-induced reductions in plant productivity in low-diversity or monoculture systems play important roles on overall plant yield [28]. Pathogen accumulation is thought to directly lead to the main symptoms of root diseases [29, 30]. Plant species-specific soil fungi, such as *Phytophthora*, *Pythium*, and *Cylindrocarpon*, seriously restrict the replant growth of numerous crops [31–33] by triggering root and rust rot. Soil fungi and nematodes can also form synergistic or antagonistic interactions with microbes. For example, *Fusarium* and nematodes can form *Fusarium*–nematode complexes that strengthen their crop-destructive power [34]. Alternatively, some bacteria (such as *Bacillus* and *Pseudomonas*) can act as biocontrol agents against pathogenic fungi [29, 35, 36]. It is, however, difficult to draw effective conclusions by studying the changes in certain types of microbes related to RD owing to the extremely

complex interactions among microbes. This highlights the need for further investigations of soil microbiome changes.

Furthermore, nutrients, metabolites, and microbes are closely correlated to each other and are dynamically transformed by natural processes; therefore, analyzing their individual and interactive effects remains challenging. In addition, neither the interaction between these three factors nor the factor with the greatest and longest-lasting effect on RD have been identified in previous research. Hence, the issues to be prioritized for minimizing its impact warrant further study.

Panax quinquefolius (American ginseng) is a perennial herb with considerable medicinal value, boosting the immune system and exerting detoxifying, antioxidant, and anticancer effects [37–39]. In addition, this plant has been used to treat severe acute respiratory syndrome, H5N1 influenza, and COVID-19 [40, 41]. The global ginseng market is predicted to reach US \$12.6 billion by 2027, and 7,033 tons of *P. quinquefolius* is expected to be sold between 2019 and 2024 [42]. *P. quinquefolius* is known to experience strong RD. The persistence of RD—often more than 20 years and even up to 80 years—is longer in this species than in other crops [1]. This limits the land available for *P. quinquefolius* cultivation. Crop rotation is often used to reduce the impacts of RD and other stressors in crops [43]. Previous studies on RD in *P. quinquefolius* have predominantly focused on the changes in soil properties of the first crop (generally cultivated for four years) [1, 44, 45] while data for long-term *P. quinquefolius*-cultivated soil samples with a history of rotation are lacking. As a result, our understanding of the nutrient-, metabolome-, and microbiome-related mechanisms that influence RD duration remains limited.

In order to further our understanding of RD we conducted field investigations in the Wendeng District of Weihai, China, aimed to analyze plots with varying cultivation and rotation histories (Supplementary Table 1). We used a space-for-time soil sequence representing common crop rotation at 1, 10, and 20 years since the cultivation of *P. quinquefolius* to explore aspects of RD in this species. Using multiomics and bioassay experiments, we examined the replanting capacity of *P. quinquefolius*-cultivated soil samples, confirmed the persistence of RD, and explored the dynamics of the biotic and abiotic properties of these soils. Specifically, we addressed the following questions. (1) Has RD or the legacy effect of *P. quinquefolius* persisted over 20 years? (2) How do the changes in nutrients, metabolome, and microbiome in *P. quinquefolius*-cultivated soils contribute to the development of RD? (3) Which of the three key factors (nutrients, metabolome, and microbiome) exerts the most prominent influence on the long-term effects of *P. quinquefolius* RD?

Methods

Study area

The study site was located in the Wendeng District of Weihai City, Shandong Province (36.87°–37.38°N, 121.72°–122.32°E). This region of China experiences a continental monsoon climate, with an annual

average temperature and precipitation of 11.9°C and 816.2 mm, respectively, and has a long history of *P. quinquefolius* cultivation. The total area of the entire study covers three-quarters of the Wenden District.

Soil sampling

We collected soil samples in April 2019 from the following sample plots: (1) farmland in which *P. quinquefolius* had never been planted (control, CK); (2) farmland with traditional crops rotated for 1 year after the *P. quinquefolius* harvest (*P. quinquefolius* was harvested in autumn and soil was sampled the following spring; A1); (3) farmland with traditional crops rotated for 10 years after the *P. quinquefolius* harvest (A10); and (4) farmland with traditional crops rotated for 20 years after the *P. quinquefolius* harvest (A20). We obtained 10 replicates for each sample type (i.e., from 10 different farmland). Each sampling plot was rotated using common crops in the area, and the manual management was similar among the treatments. Each plot was approximately 20 × 50 m in size. The farmland longitude, latitude, altitude, and information regarding aboveground crops and previous crops at the time of sampling are summarized in Supplementary Table 1.

Multi-point random sampling (following a Z-shaped sampling method) was adopted, with over 10 sampling points for each plot. After removing the litter and large gravel from the surface, approximately 100 g of soil (0–20 cm depth) was collected, and the samples were evenly mixed. Approximately 15 g of soil was transferred into cryogenic vials and preserved in a liquid N tank and dry ice box for microbiome and metabolome analyses, respectively. The vials were stored at – 80°C in the laboratory. Finally, approximately 50 kg of soil was collected from multiple points across the treatments for a pot experiment.

Plant–soil feedback pot experiment

Field-collected soil was transported back to an artificially shaded area in Wendeng where it was mixed and filled into terracotta pots (depth = 35 cm, top diameter = 40 cm, bottom diameter = 22 cm). Four pots were filled with the same soil from each plot (A1, A10, A20, and CK). One *P. quinquefolius* seedling was planted in each pot, yielding an experimental design with 4 soil types × 10 plots × 4 pots × 1 seedling, giving 160 pots. The pots were covered with wheat (*Triticum aestivum*) straw (Supplementary Fig. 1). We invited local professional planters to carry out daily management (weeding and watering in case of drought). The growth of *P. quinquefolius* seedlings was recorded every 20 days after planting. Seedlings that died within 3 weeks of transplantation were replaced with new two-year-old *P. quinquefolius* seedlings. The total yield, plant height, and crown width were measured at the *P. quinquefolius* harvest time, which was 12 weeks in this experiment.

Soil metabolomic analysis and microbiome analysis

Soil metabolomic and microbiome analyses are described in the Supplementary Materials and Methods.

Validation experiments

We carried out Petri dish and pot validation experiments on organic nutrients and increased harmful metabolites. A detailed description is provided in the Supplementary Materials and Methods.

Statistical analysis

The log-rank (Mantel–Cox) test was conducted in GraphPad Prism 8 (<https://www.graphpad.com/>) for the plant–soil feedback pot experiment to determine the differences in the survival rates of *P. quinquefolius* seedlings. Data on *P. quinquefolius* height, crown width, and biomass for each sample group; soil physicochemical properties; contents of phenolic acids and other metabolites; α -diversity indices (observed mean species richness, Shannon, abundance-based coverage estimator, and Chao indices); relative abundances of microbial taxa (bacteria, fungi, and non-fungal eukaryotes); seed germination potential; germination rate; and allelopathy index were compared using analysis of variance (ANOVA) with Duncan's multiple range tests. Statistical significance was set at $p < 0.05$, and these statistical tests were performed using SPSS v25.0 and R v4.0.5.

To clarify how soil biotic and abiotic factors respond to the extension of crop-rotation years and identify the interactions between significant potential factors (SPFs), we divided the identified SPFs according to persistence, created a correlation heatmap, and performed interaction network analyses. We selected 110 SPFs with significant differences between the A1 and CK groups. According to their persistence, these SPFs were divided into the following categories: (1) short-term SPFs (A1 vs. CK were significantly different, whereas A10 vs. CK and A20 vs. CK were not); (2) medium-term SPFs (A1 vs. CK and A10 vs. CK were significantly different, whereas A20 vs. CK were not); and (3) long-term SPFs (A1 vs. CK, A10 vs. CK, and A20 vs. CK were significantly different).

PLS–DA was used to discriminate the metabolite composition differences among the different *P. quinquefolius*-cultivated soil samples. Pearson's correlation analysis was conducted using R v4.2.5 (igraph) to identify the correlations among the SPFs. We performed network analysis using Spearman's rank correlation coefficients in R v4.2.5 to further examine the possible “collaborative” or “antagonistic” relationships among the different factors. We constructed a partial least squares structural equation model (PLS–SEM) to explain the contribution of latent variables to *P. quinquefolius* replanting survival rate (using the survival rate of *P. quinquefolius* seedlings on day 57 as the response index) in R v4.2.5. We selected 24 (3×8) observed variables (significant potential RD factors) under three latent variables (nutrients, metabolites, and microbes) based on factor analyses and existing sample size ($n = 40$).

Results

Persistence of *P. quinquefolius* RD

The replanting ability of *P. quinquefolius* in A1 soil was the poorest, showing a strong negative legacy effect associated with the lowest survival rate and total yield alongside root rot disease symptoms (Fig. 1a–f). The survival rate, total yield, and crown width of *P. quinquefolius* plants were partially restored with the extension of rotation time (A10 and A20); however, the individual average biomass remained

lower than that of CK plants. These findings indicate that *P. quinquefolius* RD can persist for 20 years, and the negative feedback effect of *P. quinquefolius* was gradually but not completely alleviated. There was no significant difference in average biomass, height, or crown width between the CK and A1 plants (Fig. 1g-i).

Acidification and reduced dissolved organic nutrients in *P. quinquefolius*-cultivated soil

The pH range suitable for *P. quinquefolius* growth is neutral to weakly acidic [46]. *P. quinquefolius*-cultivated soil acidification in the experimental soils led to unsuitable pH levels (CK = 4.69 ± 0.07 , A1 = 4.19 ± 0.04 , A10 = 4.49 ± 0.08 , and A20 = 4.34 ± 0.04 ; Supplementary Table 2). Among the measured inorganic nutrients, only the available potassium (AK) showed significantly increased content in the A1 group compared with that in the CK group (Supplementary Table 2). Increases in AK content may be caused by the application of K fertilizer owing to the high demand for potassium fertilizer by 3- to 4-year-old *P. quinquefolius* plants [47, 48]. There were no significant differences in the contents of other inorganic nutrients among the tested soils. This indicated that the RD associated with *P. quinquefolius* was not heavily influenced by inorganic nutrients. The soil contents of 11 amino acids, 12 carbohydrates, and alditols were significantly ($p < 0.05$) lower in the A1 group than in the CK group (Fig. 2a, b), and those of eight amino acids, five carbohydrates, and alditol were lower in the A20 group than in the CK group (Supplementary Table 3). The levels of two amino acids and one alditol were elevated in the A1 group relative to those in the CK group, and the aspartate level was higher in all other *P. quinquefolius*-cultivated soil samples (Fig. 2a, b and Supplementary Table 3).

Detrimental changes in the metabolite content of *P. quinquefolius*-cultivated soil

Fourteen phenolic acids were investigated using high-performance liquid chromatography (HPLC). Of these, the levels of coumaric acid, protocatechuic acid, syringic acid, p-coumaric acid, ferulic acid, and benzoic acid were lower in the A1 group than in the CK group (Supplementary Table 4), whereas the levels of syringic acid and p-coumaric acid were lower in all *P. quinquefolius*-cultivated soil groups than in the CK group. A total of 205 substances were detected using gas chromatography–mass spectrometry. Partial least squares discriminant analysis (PLS–DA) revealed that the composition and content of metabolites in the *P. quinquefolius*-cultivated soil were significantly different from those in the CK group (Fig. 3a). ANOVA revealed 56 differentially expressed metabolites in the CK and *P. quinquefolius*-cultivated soil groups. Overall, there were 23 amines (seven monoamines, six polyamines, and 10 amides), 20 carboxylic acids (three aromatic carboxylic acids with benzene rings, nine fatty acids, six carboxylic acids with a hydroxyl group, and two general carboxylic acids), two lipids, three alkanes, three pyridines, one inorganic acid, one ketone, two phenols, and one chlorinated organic compound that were differentially expressed between the CK and *P. quinquefolius*-cultivated soil groups (Fig. 2; classification

and specific relative contents are provided in Supplementary Fig. 4 and Supplementary Table 5, respectively).

The concentrations of two phenols (Fig. 2e) and three aromatic carboxylic acids (Fig. 2d) were lower in all *P. quinquefolius*-cultivated soil groups than in the CK group (Supplementary Table 5). These results, combined with those of the HPLC analysis indicate that phenolic acids do not accumulate in the soil and do not result in allelopathic inhibition. Most monoamines, polyamines, and amides were significantly depleted in the A1 group compared with those in the CK group; the levels of some (including ethanolamine, hydroxylamine, methoxyamine, cyanuric acid, oleamide, phosphodiimidic amide, and putrescine) were not restored to the CK levels even in the A20 group (Fig. 2c). The contents of some carboxylic acids with alcohol hydroxyl groups (such as 2-hydroxypentanoic acid, citric acid, erythronic acid, and lactic acid) were reduced in the A1 group compared with those in the CK group (Fig. 2d). The levels of some esters (xylonolactone and monomyristin), total alkanes (Fig. 2f), and pyridine compounds 1,3-dihydropyridine and nicotinamide (Fig. 2e) were also significantly lower in the *P. quinquefolius*-cultivated soil groups than in the CK group.

The levels of seven of the nine measured fatty acids were elevated in the A1 group, and those of myristic, decanoic, dodecanoic, and montanic acid were significantly elevated in the A1 and A20 soil samples compared with those in CK soil. In contrast, palmitic acid (C16) and stearic acid (C18) levels were significantly reduced in the A1 and A20 soils compared with those in CK soil. Pyruvic acid (Fig. 2d) and chlorphentermine (Fig. 2e) levels were significantly higher in all experimental soils than in the CK soil. The amide (-NH_2), benzene ($\text{-C}_6\text{H}_5$), alkane ($\text{C}_n\text{H}_{2n+2}$), and pyridine ($\text{-C}_5\text{H}_4\text{N}$) contents were significantly lower in the *P. quinquefolius*-cultivated soil groups than in the CK group, whereas those of most fatty acids and pyruvic acid were significantly higher in the *P. quinquefolius*-cultivated soil groups (Fig. 2).

Microbial α - and β -diversity and differences in taxa among *P. quinquefolius*-cultivated soil

The α -diversity indices of bacteria, fungi, and non-fungal eukaryotes were not significantly different among the *P. quinquefolius*-cultivated soil samples (Supplementary Fig. 5). However, the trends in the β -diversity indices did differ (Fig. 3). There were significant differences in key bacterial, fungal, and non-fungal eukaryotic taxa between the CK and *P. quinquefolius*-cultivated soil groups. The differential classification groups are described in the Supplementary Results.

Changes in beneficial and harmful microbes in *P. quinquefolius*-cultivated soil

We identified seven beneficial bacteria that act in a facilitative manner (*Sphingomonas*, *Burkholderia*-*Caballeronia*-*Paraburkholderia*, *Gemmatimonas*, *Terrabacter*, *Catenulispora*, *Mesorhizobium*, and *Methylobacterium*) as well as three fungi (*Acremonium*, *Penicillium*, and *Naganishia*) with lower abundances in the *P. quinquefolius*-cultivated soil groups relative to the CK group. Two beneficial

microbes (*Rhodanobacter* and *Acephala*) were more abundant in the *P. quinquefolius*-cultivated soil groups than in the CK group (Fig. 4a–c and Supplementary Table 6).

In addition, we observed a reduction (or no significant change) in the abundance of harmful fungi (*Fusarium* and *Cercophora*) and nematodes (phylum Nematoda, and *Meloidogyne*) in the *P. quinquefolius*-cultivated soil groups compared with that in the CK group (Fig. 4d, f, and Supplementary Table 6).

SPFs related to RD are partially restored by crop rotation

The number of SPFs decreased by 41 (mostly microbial and nutrient factors) between the short-term and mid-term categories based on this classification, whereas a large number of SPFs were included in the long-term category (Fig. 5a). The short-term (49), medium-term (8), and long-term (53) SPFs accounted for 44.55%, 7.27%, and 48.18% of the total number of factors (110), respectively. This indicated that over half (51.82%) were restored after 20 years of rotation, whereas the others (48.18%; 29 metabolites, 14 organic nutrients, 9 microbes, and pH) were not (Fig. 5a).

Strong interactions between abiotic SPFs (nutrients and metabolites)

The correlations among the abiotic factors (nutrients and metabolites) were higher than those among the biotic factors ($|R| > 0.5$), $p < 0.05$; Fig. 5b, c, Supplementary Figs. 9 and 10). We discovered positive correlations among nutrient levels and metabolite factors (abiotic factors), whereas some microbial factors and some abiotic factors only exhibited relatively weak correlations. Alanine, adenosine-5-monophosphate (Supplementary Fig. 10a), putrescine, N-acetylornithine, glyceryl, 5-aminovaleric acid, hydroxylamine, nicotinamide, histidine, pyroglutamic acid, methoxyamine, 1,2,4-benzenetriol, and stearic acid (Fig. 5b) should be researched further, given their high correlations.

Long-term duration of *P. quinquefolius* RD is mainly caused by soil metabolic factors, not pathogens

Functional prediction analysis of bacteria, fungi, and non-fungal eukaryotes showed no significant difference in bacterial community function among the four groups (Supplementary Fig. 11). The relative abundance of pathogens decreased in the A1 group compared with that in the CK group (Fig. 6a, b). However, *P. quinquefolius* still showed a high incidence of root rot under the low-pathogen condition of A1 (Fig. 1) and a low incidence under the higher pathogen condition of CK. This suggests that pathogens are the direct cause of root rot, while other mechanisms regulate disease occurrence in *P. quinquefolius* plants. Hence, there might be metabolic factors that weaken the *P. quinquefolius* resistance to pathogens that lead to a higher disease prevalence, even under low-pathogen-load conditions.

There were 35 metabolic pathways that were significantly different in the A1, A10, and A20 groups compared with the CK group. Additionally, there were 20 metabolic pathways with significant differences

in A1 and that were similar to either A10 or A20 when compared to the CK group. These results show that 18 of the 55 metabolic pathways involved glycine, 9 involved tyrosine, and 8 involved glucose (Fig. 6c). Only fatty acid biosynthesis and prodigiosin biosynthesis pathways underlie the metabolism of dodecanoic acid, both of which were upregulated in the *P. quinquefolius*-cultivated soil groups. Furthermore, 53 other metabolic pathways were downregulated in the *P. quinquefolius*-cultivated soil groups compared with their regulation in the CK group (Supplementary Table 7).

Furthermore, we constructed a PLS-SEM to explain the different replanting capacities of *P. quinquefolius*-cultivated soil. The effects of crop rotation restoration years on nutrients and metabolites were negative, whereas those on microbes were positive. These effects were larger in long-term models than in short-term models. These results indicate that long-term nutrient and metabolite factors would not be restored with the extension of rotation time and would gradually become more negative, while long-term microbial factors would have a better restoration effect than nutrient and metabolite factors, which was consistent with the results in Fig. 4a. The effects of nutrients and microbes on the survival rate in the L (long-term) model were reduced compared with those in the SM (short- and medium-term) model, whereas metabolites had the opposite effect. These results imply that nutrients and microbes that were positively affected by crop rotation and were rapidly restored are important for *P. quinquefolius* replanting in the SM model, whereas metabolites and pH that were not fully strongly restored affected replanting survival rates in the L model. In contrast, the impact of microbes on nutrients was negative in the L model. This suggests that long-term microbial factors were not conducive to long-term nutrient recovery and may serve as nutrient consumers. The positive influence of nutrients on metabolites suggests that an increase in nutrients is beneficial to metabolic factors. This is supported by the observed significant differences in metabolic pathways mostly involving glycine, tyrosine, and glucose.

SPF validation

Beneficial effects of dissolved organic N (amino acids) on P. quinquefolius seedling growth Although the amino acid content in *P. quinquefolius*-cultivated soil decreased significantly, their effects on *P. quinquefolius* growth are unknown. Glycine and tyrosine are involved in multiple metabolic pathways (Fig. 7c); their levels were significantly reduced and, thus, they were selected as artificially added organic N sources [49–51]. Natural soils containing only organic N are rare. Therefore, we conducted separate and mixed treatments with inorganic and organic nutrients. We selected two forms of organic N (glycine and tyrosine) and inorganic N (ammonia N [AN] and nitrate N [NN]) nutrients for the bioassay of *P. quinquefolius* seedlings. AN inhibited the growth of *P. quinquefolius* to a greater extent than NN. This suggests that *P. quinquefolius* plant growth benefits from NN. The separate addition of organic nutrients had no significant effect on *P. quinquefolius* growth; however, mixing organic and inorganic N (NN:AN = 3:1) significantly improved *P. quinquefolius* growth (Fig. 7).

Detrimental effects of increased pyruvic acid levels on P. quinquefolius growth Low pyruvic acid concentrations (0.1 mmol L^{-1}) promoted the germination of *P. quinquefolius* seeds, whereas high concentrations ($0.5\text{--}5 \text{ mmol L}^{-1}$) had inhibitory effects (the comprehensive allelopathic effect index is

listed in Supplementary Table 8). Pot experiments with two pyruvic acid concentrations (0.5 and 2.5 mmol L⁻¹) showed that the growth of *P. quinquefolius* was mostly unaffected when it was grown in a vermiculite-based growth medium, whereas a significant inhibitory effect was noted in a soil-based medium (Fig. 7 and Supplementary Fig. 12).

Detrimental effects of increased fatty acid levels on P. quinquefolius growth Fatty acids, myristic acid, and octanoate at concentrations of 7.00 mmol L⁻¹ significantly inhibited the germination of *P. quinquefolius* seeds. Decanoic acid (7.00 mmol L⁻¹) also significantly inhibited the germination potential of *P. quinquefolius* seeds; however, it did not inhibit the germination rate. When we used mixed fatty acids at variable concentrations (0.44–7.00 mmol L⁻¹), the germination potential and rate were significantly reduced in a dose-dependent manner (Supplementary Table 9).

We also compared the effect of two concentrations of mixed fatty acids (0.44 and 1.75 mmol L⁻¹). The leaf expansion rate of *P. quinquefolius* in a vermiculite-based medium (CK = 100%, FA0.44 = 75%, and FA1.75 = 50%) was considerably affected by fatty acids, with several seedlings failing and presenting small and deformed leaves (Supplementary Fig. 13). The other parameters did not differ between the two fatty acid concentration groups. When a plant was grown in a soil-based medium, leaf expansion was significantly inhibited (Fig. 7 and Supplementary Fig. 12).

Discussion

We explored the legacy effect on *P. quinquefolius* replanting and observed that crop rotation could promote the *P. quinquefolius* replanting capacity in *P. quinquefolius*-cultivated soil to some extent. However, the RD or legacy caused by *P. quinquefolius* remained detectable even after 20 years of rotation cultivation based on analyses of average biomass and 53 long-term SPFs. Our results provide novel insights into the mechanisms and key drivers of RD in *P. quinquefolius*.

Plants with long non-growing seasons (such as alpine and tundra plants) have a stronger preference for soil-dissolved organic N (amino acids) than for inorganic N [14]. Small-molecular-weight carbohydrates and alditol can be effectively absorbed by plants [16] that may influence N absorption [51]. We confirmed that a mixed treatment of amino acids and inorganic N significantly improved *P. quinquefolius* growth. Hence, a reduction in levels of these amino acids and small-molecular-weight sugars in *P. quinquefolius*-cultivated soil could limit *P. quinquefolius* replanting success. We also found that *P. quinquefolius* preferred nitrate N over ammonia N. Similar phenomena were reported for other *Panax* spp. [52]. Therefore, we suggested that *Panax* prefers nitrate N over ammonia N.

We confirmed that pyruvic acid and fatty acids act as allelochemicals that directly inhibit *P. quinquefolius* seed germination and seedling growth. Pyruvic acid was negatively correlated with pH (Fig. 5c, $R = -0.5$, $p < 0.01$), since it can ionize H⁺ in solution (as well as the aspartate in *P. quinquefolius*-cultivated soil) that aggravates soil acidity. Furthermore, we observed a decrease in phenolic acid content in *P. quinquefolius*-cultivated soil. Multiple phenolic acids can inhibit the proliferation of *P. quinquefolius* pathogens at high

concentrations but had no effect or promoted the proliferation of *P. quinquefolius* pathogens at low concentrations [53, 54]. Thus, reduction in the phenolic acid content of *P. quinquefolius*-cultivated soil weakens this inhibitory effect on pathogens and may even promote pathogen growth. Notably, the artificial addition of *P. quinquefolius* root residues can decrease the phenolic acid content in soil [55]. The seasonal loss of *P. quinquefolius* fibrous roots (similar to the addition of residues) may contribute to this phenomenon in *P. quinquefolius*-cultivated soil.

The amine content in the soil was reduced after *P. quinquefolius* cultivation. Hydroxylamine (monoamine) is involved in the oxidation and reduction of ammonium in soil [56], ATP acquisition, and N metabolism [27]. Hydroxylamine levels were lower in the *P. quinquefolius*-cultivated soil groups than in the CK group, which may be detrimental to *P. quinquefolius* replanting. Similarly, ethanolamine (monoamine) protects plants from stress [26, 57]. Amide cyanuric acid serves as an indicator of soil fertility and participates in the metabolic processes of atrazine [58, 59], including downregulation of ko00791 identified in this study (Supplementary Table 7). Cyanuric acid can also be converted into ammonia and carbon dioxide by *Pseudomonas* sp. to provide nutrients and raw materials for photosynthesis [60]. The decline in cyanuric acid levels in *P. quinquefolius*-cultivated soil represents a lack of soil fertility that is consistent with the lack of dissolved organic nutrients. Polyamines can increase crop stress resistance [61]. For example, putrescine can alleviate salt and chilling stress in *Brassica juncea* [62] and watermelon [63], and agmatine improves plant disease resistance [64]. We observed reduced pyridine content in *P. quinquefolius*-cultivated soil. Nicotinamides are growth regulators affecting several plant physiological processes, such as enzyme activity and nucleic acid protein biosynthesis [65], which have been shown to improve the growth of broad beans [66]. As a consequence, a reduction in nicotinamide content observed in *P. quinquefolius*-cultivated soil may weaken its regulatory effect on *P. quinquefolius* growth. Upregulated fatty acid biosynthesis pathways suggest a gradual accumulation of fatty acids in the *P. quinquefolius*-cultivated soil groups, which eventually adversely affect *P. quinquefolius* growth. Furthermore, 53 other metabolic pathways were downregulated in the *P. quinquefolius*-cultivated soil groups compared with their regulation in the CK group. This indicates that the overall metabolism of *P. quinquefolius*-cultivated soil was disordered. The degradation of harmful substances associated with *P. quinquefolius* cultivation and the recovery of beneficial substances were hindered in *P. quinquefolius*-cultivated soil. In summary, reports on the roles of other metabolites and metabolic pathways in *P. quinquefolius*-cultivated soil are rare; however, the observed changes in the metabolome associated with *P. quinquefolius*-cultivated soil are not conducive to *P. quinquefolius* replanting.

There was no significant difference in the α -diversity indices of bacteria, fungi, and non-fungal eukaryotes among the CK and *P. quinquefolius*-cultivated soil. Hannula et al. [67] experimentally confirmed that the soil microbiome legacy of the previous plant diminishes over time, whereas the effects of the current plant on the soil microbial community concomitantly increase with time. The *P. quinquefolius*-cultivated soil in our study was rotated with wheat, corn, and other common local crops. The rotation of aboveground vegetation might limit the differences in microbiome α -diversity. In contrast, the β -diversity indices of bacteria, fungi, and non-fungal eukaryotes were significantly different among the CK and *P. quinquefolius*-cultivated soil, and some microbes could maintain microbial legacy of *P. quinquefolius* for

20 years. This may be attributed to the perennial nature of *P. quinquefolius* (generally cultivated for 3–5 years) that might lead to prolonged effects on the soil.

The changes in the abundance of beneficial and harmful organisms and the prediction of microbial function indicate that pathogen accumulation was not the root cause of RD. Furthermore, the substantial reduction in the number of beneficial microbes observed in the *P. quinquefolius*-cultivated soil samples was related to the nutrients (Supplementary Table 6; the relative abundances of *Gemmatimonas* and *Terrabacter* were positively correlated with organic nutrients) and can also indirectly affect the soil metabolome (Fig. 6c). Therefore, although clarifying the complex interaction between nutrients, metabolome, and microbiome factors remains challenging, static analyses of the influence of these three factors on *P. quinquefolius*-cultivated soil indicate that metabolome changes have the greatest impact on *P. quinquefolius* RD.

This study had some limitations. First, the methods to reduce the levels of pyruvic acid and fatty acid in *P. quinquefolius*-cultivated soil remain unresolved. Second, strategies to improve the overall metabolism of *P. quinquefolius*-cultivated soil warrant further investigation. Lastly, the potential positive correlation between beneficial microorganisms and nutrients, along with the potential restorative role of soil microorganisms, poses important questions that should be tackled in future research.

Conclusions

Our study demonstrates that the soil legacy effects (or intraspecific negative plant–soil feedbacks) of perennial plants can persist for 20 years or more. While the microbial legacy can diminish and be restored to the non-cultivated level of the plant with crop rotation, the nutrient and metabolite legacy in the soil may last for over 20 years. Furthermore, overall changes in the levels of nutrient, metabolite, and microbial factors contributed to the short-term (1–10 years) persistence of RD, whereas the metabolome changes were the main cause of RD in the longer term (20 years). Our study helps to better understand the relationship between RD and its potential driving factors caused by the soil legacy effects of some valuable medicinal plants, and provides theoretical guidance for effective soil improvement.

Abbreviations

HPLC

high-performance liquid chromatography

RD

replant disease

SM

short- and medium-term

SPF

significant potential factors

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The raw sequencing data generated in this study are available in the Sequence Read Archive at the National Center for Biotechnology Information under the Bioproject ID PRJNA 922374.

Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

C.L. and Y.H. conceived and designed the research. C.L., G.C., and N.Z. performed the experiments and collected the data. C.L. and G.C. wrote the main manuscript. C.L., N.Z., H.L., P.Z., X.B., and YH designed and performed the data analyses. H.Z., Y.H., Y.Z., and X.Z. reviewed and polished the manuscript. All authors participated in the data interpretation and reviewed the manuscript.

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Figures

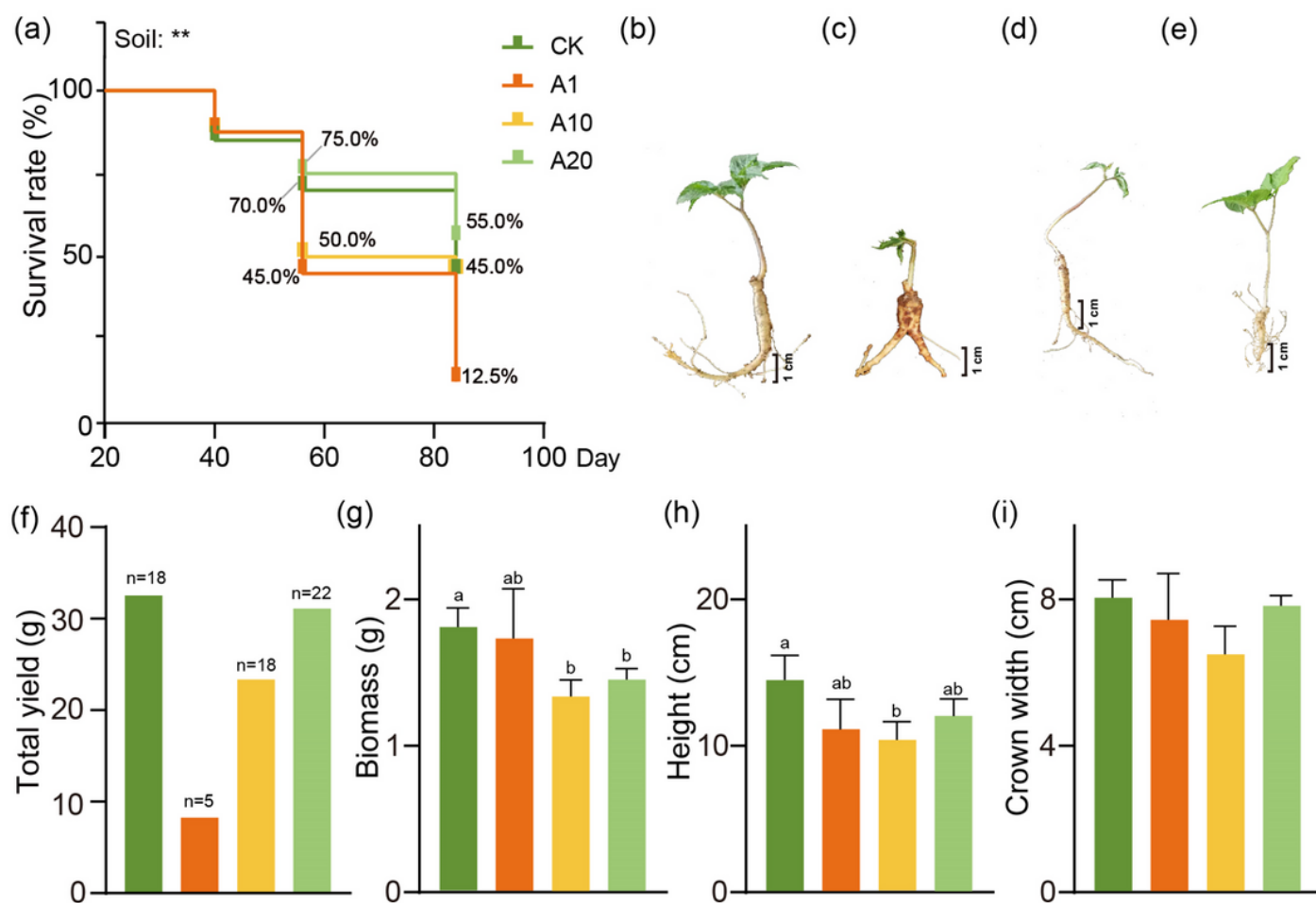


Figure 1

Panax quinquefolius replant disease was gradually but not completely alleviated. (a) Replanting survival rates of *P. quinquefolius* seedlings in *P. quinquefolius*-cultivated soil ($n = 10 \text{ plots} \times 4 \text{ pots} \times 1 \text{ seedling}$); ** (log-rank Mantel–Cox test, $p < 0.01$) and different lowercase letters (one-way ANOVA, $p < 0.05$) indicate significant differences between groups. Note: Seedlings that died within 3 weeks of transplantation were replaced with new two-year-old *P. quinquefolius* seedlings. (b), (c), (d), and (e) *P. quinquefolius* seedlings following 56 days of growth in CK, A1, A10, or A20 soils, respectively. (c) *P. quinquefolius* exhibiting rust rot symptoms. (f) Total yield after 84 days in different *P. quinquefolius*-cultivated soils. (g), (h), and (i) Individual mean biomass, plant height, and crown width of *P. quinquefolius* plants after 82 days in different *P. quinquefolius*-cultivated soil samples, respectively. CK, control soil with no ginseng cultivation history; A1, soil with 1-year post-ginseng rotation; A10, soil with 10-year post-ginseng rotation; A20, soil with 20-year post-ginseng rotation.

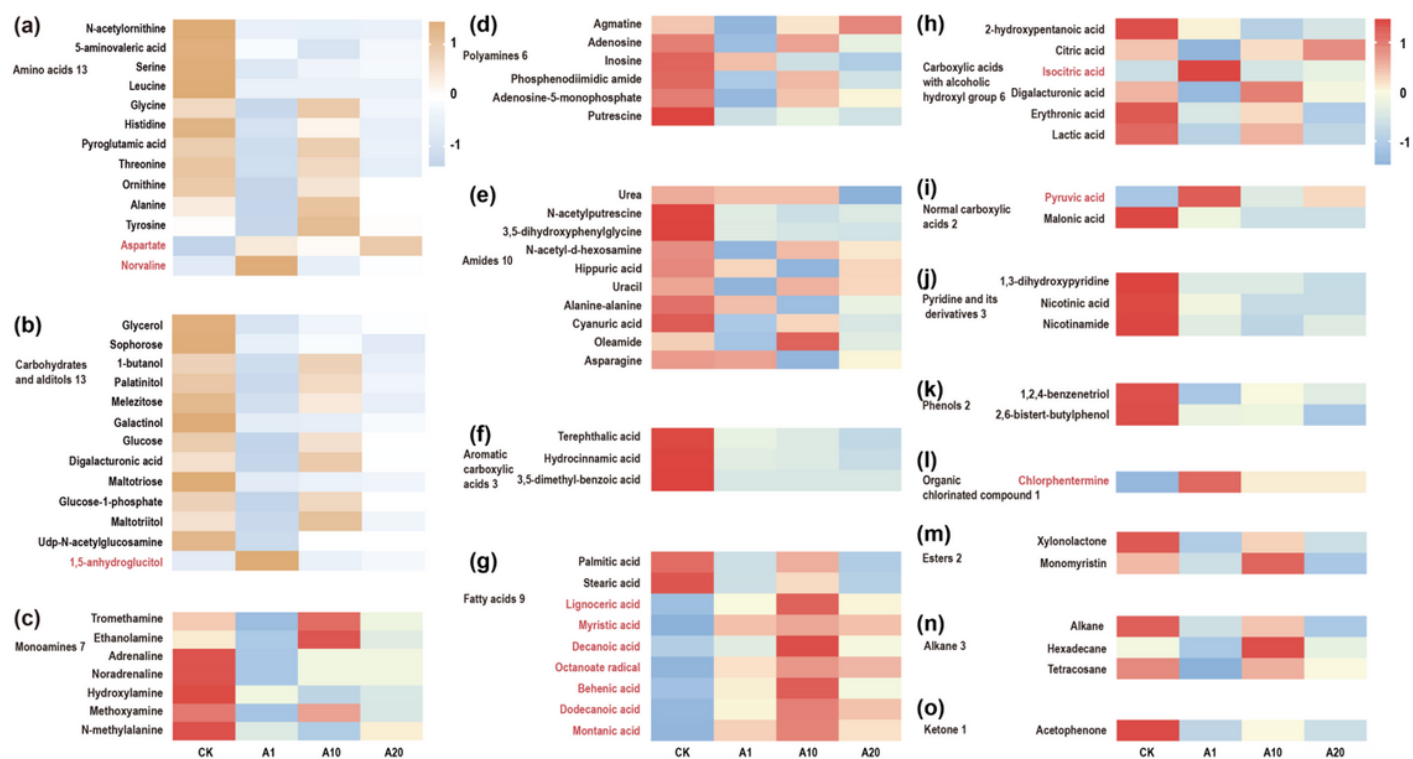


Figure 2

Heatmaps of organic nutrient and metabolite contents. (a) Amino acids. (b) Carbohydrates and alditols. (c) Monoamines. (d) Polyamines. (e) Amides. (f) Aromatic carboxylic acids. (g) Fatty acids. (h) Carboxylic acids with an alcoholic hydroxyl group. (i) Normal carboxylic acids. (j) Pyridine and its derivatives. (k) Phenols. (l) Organic chlorinated compounds. (m) Esters. (n) Alkanes. (o) Ketones. The number of molecules identified in each group follows the name. CK, control soil with no ginseng cultivation history; A1, soil with 1-year post-ginseng rotation; A10, soil with 10-year post-ginseng rotation; A20, soil with 20-year post-ginseng rotation. The yellow and red colors in the index on the right indicate high relative content, whereas blue indicates low content. The content of all substances was significantly different between the CK and A1 soils ($p < 0.05$, $n = 10$). The red font indicates that the metabolite content was increased.

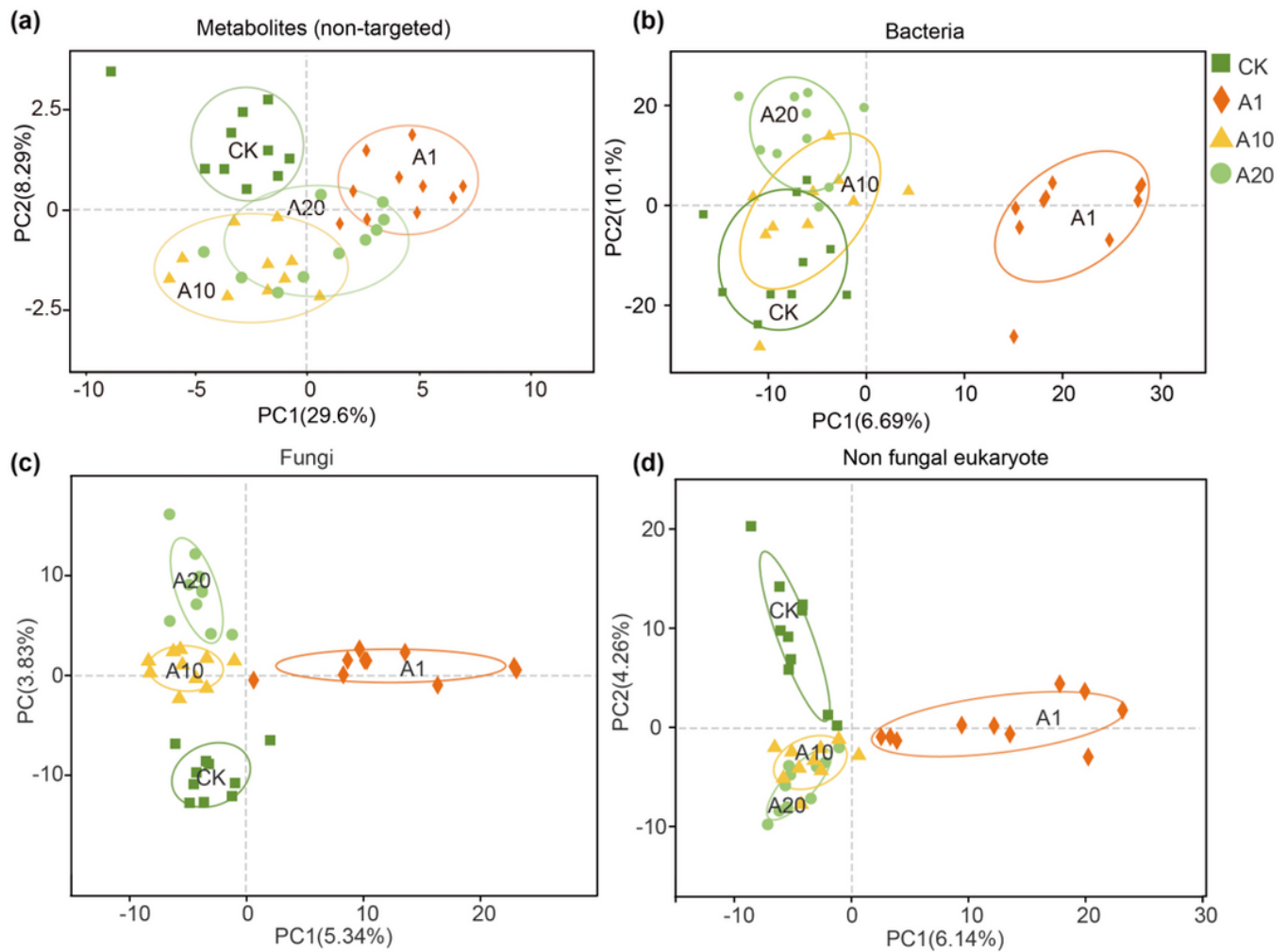


Figure 3

Soil metabolic and microbial legacy related to *P. quinquefolius* replant disease cannot be restored to the CK levels after 20 years. Partial least squares discriminant analysis (PLS-DA) of soil metabolites (a) and bacterial (b), fungal (c), and non-fungal eukaryotic (d) community structure. The shorter the distance between the two points, the higher the similarity between the two groups. CK, control soil with no ginseng cultivation history; A1, soil with 1-year post-ginseng rotation; A10, soil with 10-year post-ginseng rotation; A20, soil with 20-year post-ginseng rotation.

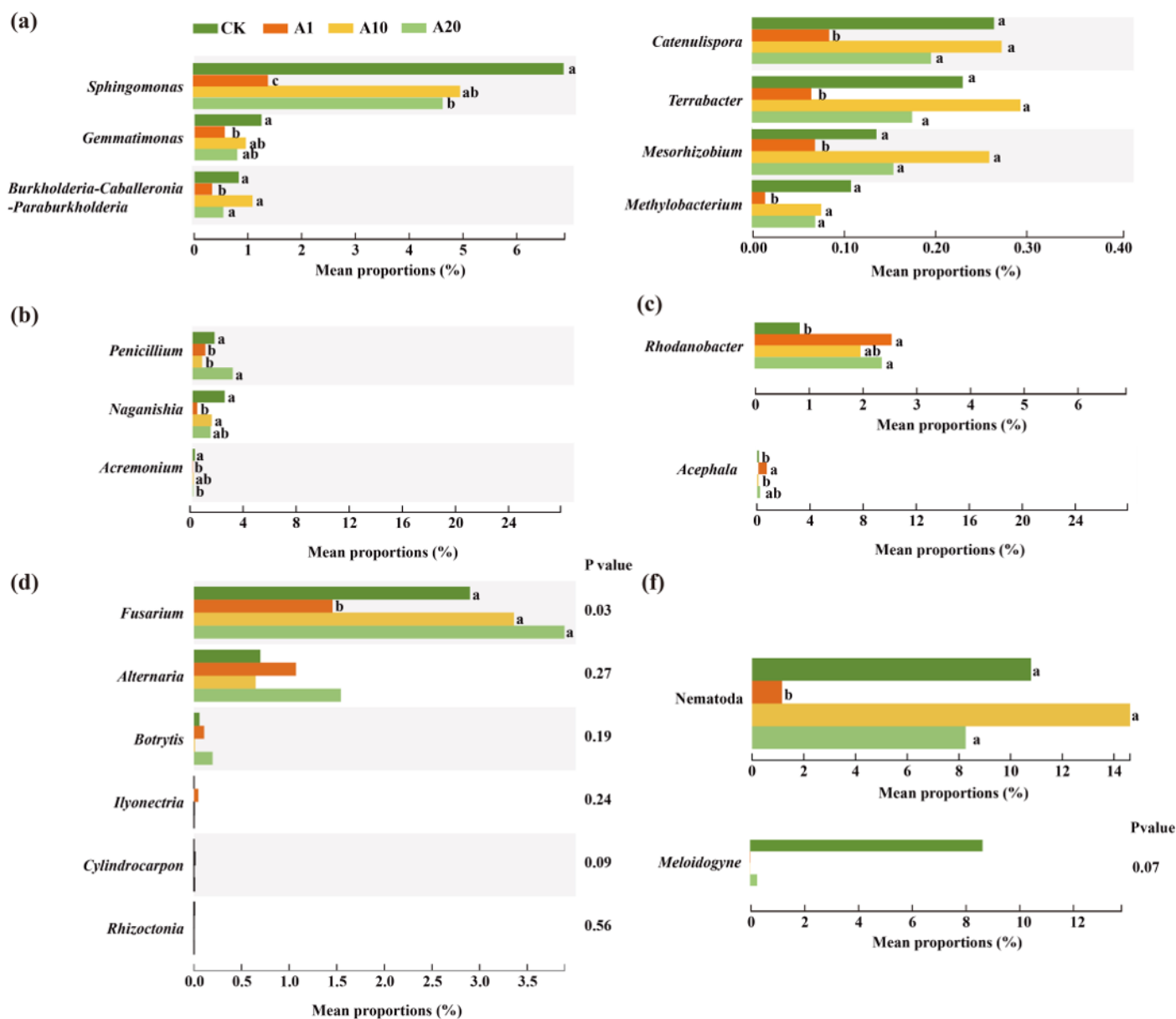


Figure 4

Changes in the abundances of functional microbes that contribute to positive and negative legacy effects. (a) Abundance of beneficial bacteria in the *P. quinquefolius*-cultivated soil. (b) Abundance of beneficial fungi in the *P. quinquefolius*-cultivated soil (c) Abundance of potentially beneficial bacteria and fungi in the *P. quinquefolius*-cultivated soil. (d) Abundance of harmful fungi in the *P. quinquefolius*-cultivated soil. (f) Abundance of harmful nematodes in the *P. quinquefolius*-cultivated soil. Note: see Supplementary Table 6 for descriptions of the relevant beneficial or harmful functions of microbes.

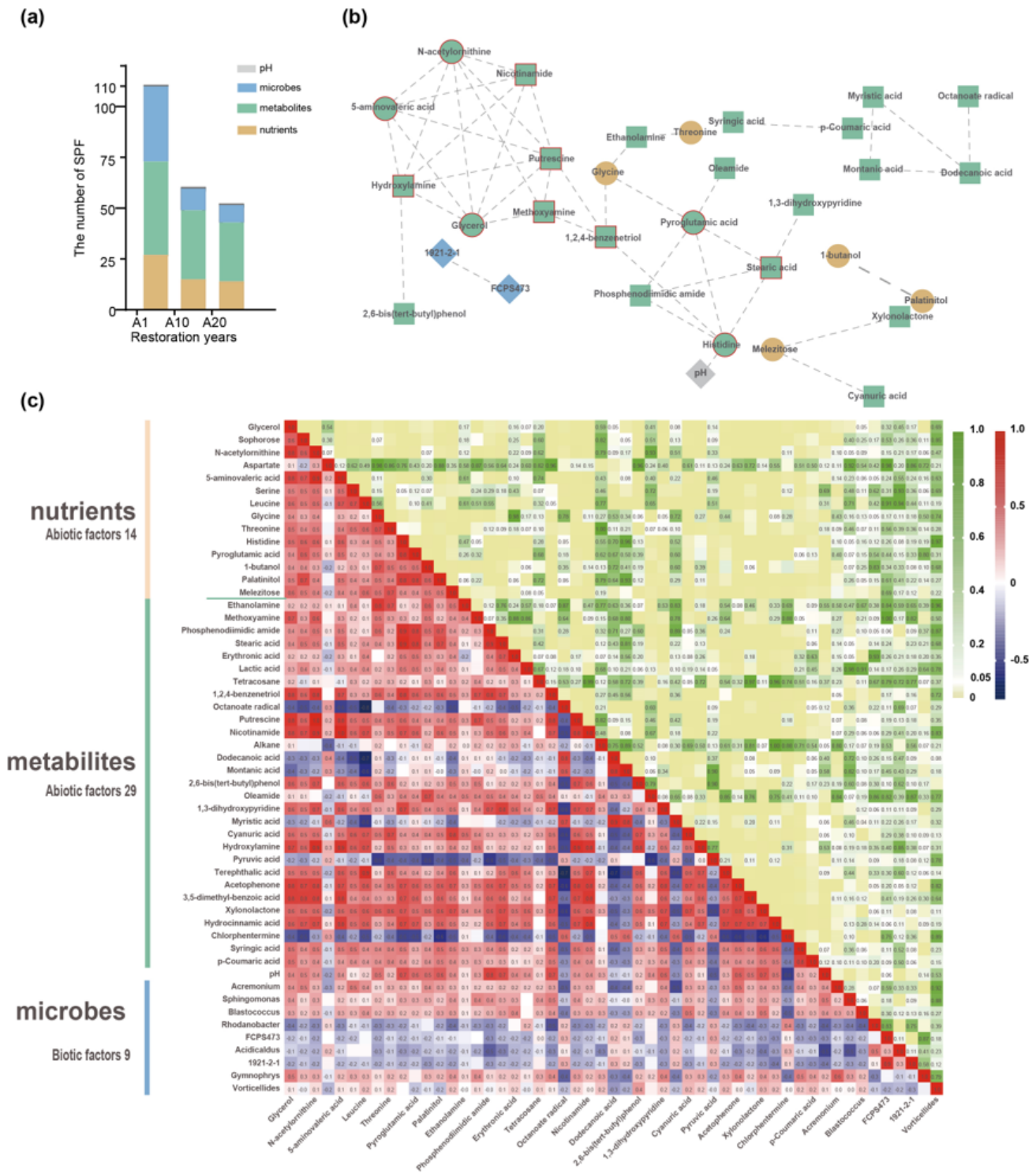


Figure 5

Number of changes and interactions of significant potential factors (SPFs). (a) Changes in SPFs among *P. quinquefolius*-cultivated soil. A1, 1-year post-ginseng rotation soil; A10, 10-year post-ginseng rotation soil; A20, 20-year post-ginseng rotation soil. (b) Network analysis of long-term SPFs in *P. quinquefolius*-cultivated soil. The correlation coefficients were calculated, whereby $|R| > 0.7$ and $p < 0.01$ constitute an association network node. The different colors and shapes identify the different types of factors (yellow

circles = nutrient factors, green squares = metabolite factors, and blue diamonds = microbial factors). A connection between nodes indicates a correlation between the two factors, and a dotted line indicates a positive correlation. The more connections pass through a node, the stronger the degree of correlation. The red lines indicate the factors with a correlation degree (number of correlated nodes) ≥ 4 . (c) Heatmaps of long-term SPFs in *P. quinquefolius*-cultivated soil. Pearson's correlation heatmap is shown on the lower left, wherein the numbers in the grid represent the correlation R-value. The correlation significance heatmap is shown on the upper right, where *p*-values are shown in the grid (*n* = 10).

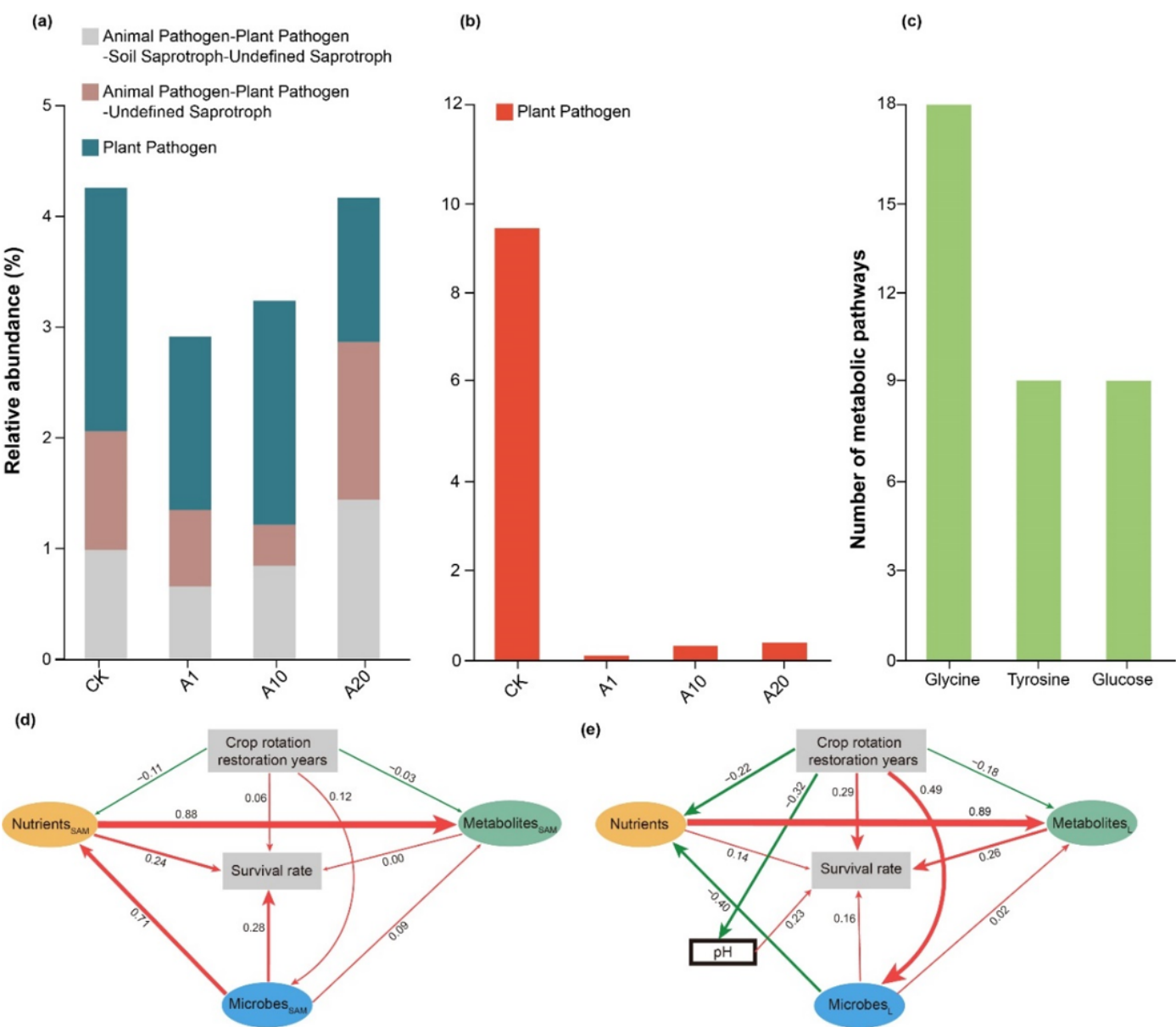


Figure 6

Long-term duration of *P. quinquefolius* RD is primarily caused by soil metabolic factors, not pathogens. (a) and (b) Functional prediction analysis of fungal and non-fungal eukaryotes. (c) Number of significantly different metabolic pathways involving glycine, tyrosine, and glucose. The partial least

squares structural equation model (PLS-SEM) was used to evaluate the effects of interactions between SPFs and *P. quinquefolius*-cultivated soil samples on the survival rate (on day 56) of *P. quinquefolius* replanting. (d) Structural equation model constructed from short- and medium-term SPFs (SM). (e) Structural equation model constructed from long-term SPFs (L). Boxes represent observed variables, while ellipses represent latent variables. The coefficients on the paths were standardized, indicating the relative importance of each variable. The line color represents positive (red) or negative (green) effects; the wider the line, the greater the effect.

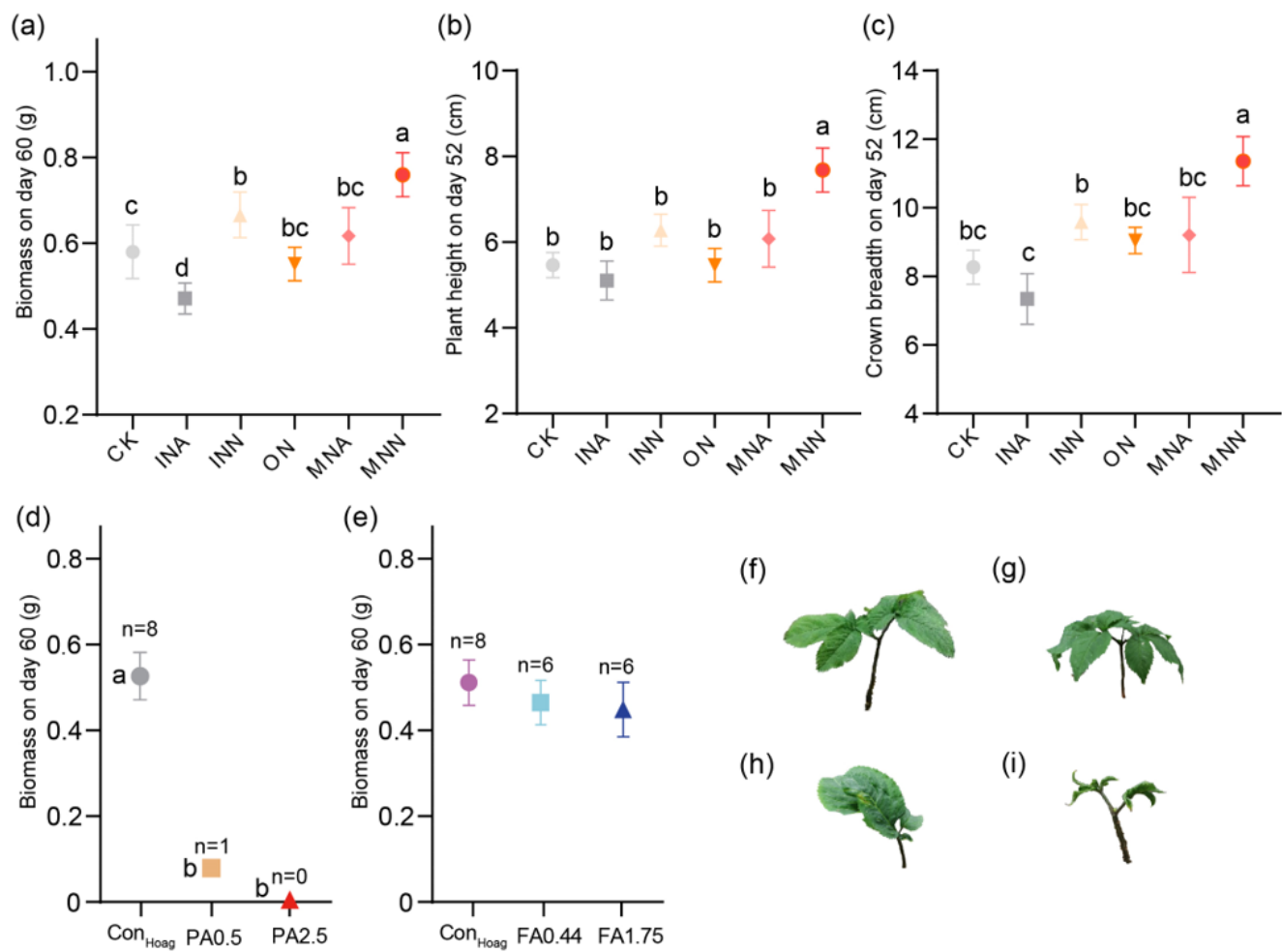


Figure 7

Validation of significant potential factors. (a), (b), and (c) Effects of dissolved organic nitrogen (N) and inorganic N on *P. quinquefolius* seedling growth. CK, normal N addition (ammonia N/nitrate N = 1/1); INA, inorganic N, with AN dominant (AN/NN = 3/1); INN, inorganic N, with NN dominant (AN/NN = 1/3); ON, only organic N (0.1 mmol L⁻¹ glycine and 0.5 mmol L⁻¹ tyrosine); MNA, mixed N, with AN dominant: ON and INA were diluted and mixed (the concentration of total N was consistent with the other treatments); and MNN, mixed N, with NN dominant: ON and INN were diluted and mixed (the concentration of the total N component was consistent with the other treatments). CK, MNA, and MNN had 20 replicates; INA, INN,

and ON had 40 replicates. (d) and (e) Effects of pyruvic acid and fatty acids on the growth of *P. quinquefolius* seedlings. Con_{Hoag} represents the Hoagland nutrient solution treatment group, whereas PA0.5 and PA2.5 represent the 0.5 and 2.5 mmol L⁻¹ pyruvic acid treatment groups, respectively; FA0.44 and FA1.75 represent the 0.5 and 2.5 mmol L⁻¹ fatty acid treatment groups, respectively. Different lowercase letters indicate significant differences (covariance analysis, $p < 0.05$). (f, g) Control group supplemented with Hoagland nutrient solution; (h, i) treatment group supplemented with mixed fatty acid solution. The figures show the leaf spreading morphology on day 13.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterial10.17.docx](#)