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Growth substrates alter aboveground plant microbial and metabolic properties thereby influencing insect herbivore performance

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The gut microbiome of plant-eaters is affected by the food they eat, but it is currently unclear how the plant metabolome and microbiome are influenced by the substrate the plant grows in and how this subsequently impacts the feeding behavior and gut microbiomes of insect herbivores. Here, we use *Plutella xylostella* caterpillars and show that the larvae prefer leaves of cabbage plants growing in a vermiculite substrate to those from plants growing in conventional soil systems. From a plant metabolomics analysis, we identified 20 plant metabolites that were related to caterpillar feeding performance. In a bioassay, the effects of these plant metabolites on insects' feeding were tested. Nitrate and compounds enriched with leaves of soilless cultivation promoted the feeding of insects, while compounds enriched with leaves of plants growing in natural soil decreased feeding. Several microbial groups (e.g., *Sporolactobacillus*, *Haliangium*) detected inside the plant correlated with caterpillar feeding performance and other microbial groups, such as *Ramlibacter* and *Methylophilus*, correlated with the gut microbiome. Our results highlight the role of growth substrates on the food metabolome and microbiome and on the feeding performance and the gut microbiome of plant feeders. It illustrates how belowground factors can influence the aboveground properties of plant-animal systems, which has important implications for plant growth and pest control.

cultivation pattern, gut microbiome, herbivory, leaf microbiome, leaf metabolome

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INTRODUCTION

The role of insect microbiomes, in particular the microbiomes of insect herbivores, in insects' feeding performance has received considerable recent attention (Callegari et al., 2020; Hammer and Bowers, 2015; Raza et al., 2020; Zhu et al., 2021). The composition of the microbial community inhabiting the gut of insect herbivores will be determined largely by the food they eat. However, a growing number of studies have shown that other, e.g., environmental factors, also influence the composition of the gut microbiome (Muratore et al., 2020). For example, it has been reported that foliar-feeding insects acquire microbiomes direct from the soil rather than the host plant (Hannula et al., 2019).

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Despite the growing body of evidence linking soil/substrate quality and the composition of soil/substrate microbiomes to plant health, there remains a paucity of evidence on how these characteristics are linked to the gut microbiomes of herbivores. Conceptually, three pathways could link soil (or substrate) microbiomes and insect microbiomes. First, the soil or substrate microbiome may influence the gut microbiome directly, as has been reported for both insects and animals (Chi et al., 2005; Hannula et al., 2019; Sugio et al., 2015). For example, Kikuchi et al. (2007) showed that *Riptortus clavatus* acquired a beneficial gut symbiont of the genus *Burkholderia* from the soil in every generation. Second, the soil or substrate microbiome and other abiotic properties affiliated with the substrate may influence the microbiome of plant tissues which then impacts the herbivore gut microbiome via the consumption of plant tissue (Hannula et al., 2019). Third, the soil microbiome may influence the nutritional quality of plant tissue, and after ingestion, the chemical composition of the food can then impact the microbial community inside the insect herbivore (Santos-Garcia et al., 2020).

Changes in the acquisition of nutrients within different cultivation systems can alter the soluble nutrient concentrations and/or the production of secondary metabolites in plant tissue (Altieri and Nicholls, 2003). Noted studies have shown that the available nitrogen in the substrate often impacts plant productivity and tissue quality (Flanagan and Cleve, 1983; Rosen and Allan, 2007; van der Heijden et al., 2008). For example, the accumulation of nitrogen-enriched compounds in the leaves can enhance insect herbivory (Altieri and Nicholls, 2003; Megali et al., 2014). The health of these plant consumers, i.e., herbivores, is influenced both directly and indirectly by the properties of the plant tissue they consume (Mattson, 1980; Ohgushi, 2005). Understanding how abiotic and biotic properties of the substrate in which the plant grows influence the nutritional and metabolome/defense characteristics of the aboveground parts and how this, in turn, influences the feeding performance of herbivore pests, remains an important question in agricultural production (Bezemer and van Dam, 2005). Plant secondary metabolites, both volatile and non-volatile, are important for the defense of the plant against insect attack (Blundell et al., 2020; Kollner et al., 2008; Pichersky and Gershenzon, 2002; Van Poecke et al., 2001). The jasmonic acid (JA) signaling pathway mediates plant defense responses that are effective against leaf-chewing insects (De Vos et al., 2005), but the induction of other pathways can also lead to changes in the plant that impact insect herbivory. Untargeted metabolomics approaches that measure a wide range of compounds can be used to detect potential metabolites involved in plant-herbivore interactions (Marti et al., 2013; Tenenboim and Brotman, 2016; Zogli et al., 2020).

In this study, we selected three substrates, including vermiculite as a soilless cultivation system and two different soil types to generate variations in cabbage leaf metabolomes and microbiomes, and then test the effects on the behavior and gut microbiome of caterpillars of the diamondback moth (*Plutella xylostella*). Additionally, in a separate exercise of bioassay, selected metabolites were assessed for their ability to influence the feeding behavior of *Plutella xylostella*. We asked the following questions: (i) how does the substrate type influence the plant microbiome and metabolome of cabbage? (ii) How does the substrate type influence the gut microbiome and feeding behavior of the herbivore? (iii) How are the metabolome and plant microbiome related to the gut microbiome and feeding behavior of this herbivore?

RESULTS

Performance and gut microbiome of caterpillars

To evaluate the influence of different cultivation systems on the feeding preferences of *Plutella xylostella*, cabbages grown in vermiculite and both soil systems (SSs) were tested. The two soils used in our study were collected from fields with a history of long-term chemical-input management (Soil 1) and organic management (Soil 2). *Plutella xylostella* fed more from plants grown in the vermiculite system than from plants grown in the two soils (Figure 1A). Insects feeding on cabbages in the vermiculite system were significantly larger (30%) than those feeding in the two soil systems (Figure 1B; Table S1 in Supporting Information). When checking the development of the remaining insects, we did not observe an obvious difference in the development rate between treatments.

To disentangle whether the gut microbiome of caterpillars differed between cultivation systems, the diversity and composition of the gut microbiome were characterized by 16S rRNA gene amplicon sequencing. Our data show that the *alpha* diversity of the larval gut microbiome was significantly (P<0.05; Wilcoxon t-test) higher when fed on plants growing in Soil 1 and Soil 2 than in vermiculate (Figure 1C; Table S2 in Supporting Information). Not only did the total gut microbial community composition differ between the vermiculate and the two soil systems (Adonis, P=0.002, R=0.741, PERMANOVA; Figure 1D), but the composition in terms of family levels also differed. *Alcaligenaceae* and *Microbacteriaceae* were the dominant families in the gut microbiome of the vermiculite system, while *Pseudonocardiaceae* was the dominant family in the gut microbiome of the two soil systems (Figure 1E). Furthermore, we conducted a microbiome network analysis to investigate the complexity and relationship of the co-occurrence microbiome. Negative correlation ratios and network properties, such as the number of edges and the average degree within the gut bacterial community of the larvae feeding on cabbage growing in the

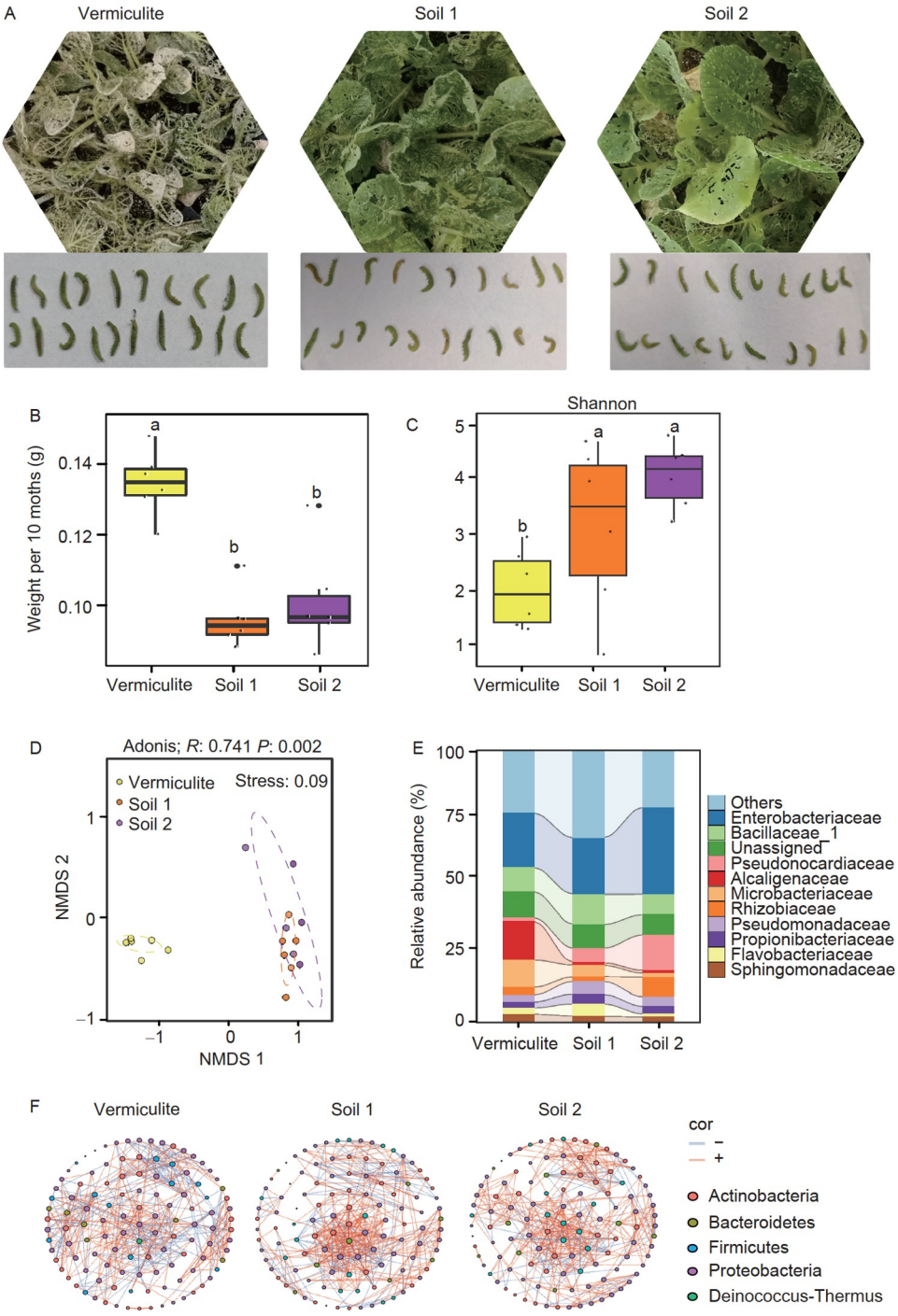

Figure 1 *Plutella xylostella* feeding behavior and gut microbial communities in the soil and vermiculite systems. A, Photos of *Plutella xylostella* feeding damage and larval sizes in the different substrates. B, Box plot showing the weight of *Plutella xylostella* feeding on the cabbage grown in the different systems. The different letters above bars represent significant differences ($P \leq 0.05$). C, Box plot showing Shannon of the gut bacterial communities in the different systems. The different letters above the columns represent significant differences ($P \leq 0.05$). D, NMDS analysis based on Bray-Curtis dissimilarities performed on the taxonomic profiles at the OTU level. *R*- and *P*-values are based on an Adonis test. E, Relative abundance (%) of the 10 major families present in the gut bacterial communities. F, Co-occurrence networks of the abundant OTUs in the *Plutella xylostella* gut microbiome in the different systems. Box plots show the median, interquartile range, 1.5× interquartile range, and outliers.

vermiculate system (VS), were less than that of the larvae feeding from plants grown in the two soil systems. Co-occurrence network analysis further indicated that the gut microbial communities in the two soil systems were more stable than those in the vermiculate system (Figure 1F; Table S3 in Supporting Information).

Leaf microbiome and metabolome

To identify the principal factors that impacted caterpillar feeding performance, leaf nutrient content and the microbiome and metabolome were analyzed. Plants growing in soil systems had significantly higher *alpha* bacterial diversity, as indicated by Chao1 and Shannon evenness, than plants growing in vermiculate (Figure 2A and B; Table S4 in Supporting Information), and the *alpha* diversity of plants growing in Soil 2 was higher than that of plants growing in Soil 1 (Figure S1 in Supporting Information). The composition of the bacterial communities also differed between the two systems (principal coordinate analysis (PCoA); Adonis, P=0.001, R=0.819 PERMANOVA; Figure 2C). Leaves of plants growing in one of the two soils had a higher relative abundance of *Firmicute*s and *Actinobacteria*, while leaves of plants growing in vermiculate had a greater abundance of *Proteobacteria* (Figure 2D). Network analysis identified more connections, especially positive connections, in the leaf microbiome of plants growing in vermiculate than of those growing in soil (Figure 2E; Table S5 in Supporting Information).

Leaf chemical properties also varied between the two soil and vermiculite systems. Higher concentrations of NO_3^- were found in leaves grown in the vermiculite system, while no significant difference was found in concentrations of the plant hormone salicylic acid (SA) between the soil and vermiculite systems (Figure 2F; Table S6 in Supporting Information). The JA level in plants grown in vermiculite differed significantly from that of plants grown in Soil 2, while the other paired comparisons were not significantly different (Figure 2F; Table S6 in Supporting Information). Analysis of the leaf metabolome by gas chromatograph coupled with a time-of-flight mass spectrometer (GC-TOF-MS) yielded a total of 2,737 chromatographic peaks, and the metabolites differed between the two systems (P=0.001, R=0.34) (Figure 2G). From the peaks, 212 compounds were identified, and these were made of 49 sugars, 17 sugar alcohols, 33 small molecule organic acids, 21 long-chain organic acids, 11 esters, 21 alcohols, 29 amino acids and amides, 5 nucleotides and 17 other compounds (Figure 2H; Table S7 in Supporting Information). The relative abundance of the 52 compounds identified in leaves in the two soil systems was significantly different (P<0.05) from the vermiculite system (Figure S2 and Table S8 in Supporting Information).

A network was constructed to illustrate correlations between the leaf metabolome, leaf chemical properties and the bacterial community inside the leaves. More edges were found in the vermiculate than in the soil systems. In Soil 1, more edges were identified between leaf chemical properties and the bacterial community, while networks belonging to Soil 2 harbored more connections between the leaf metabolome and leaf nutrients (Figure S3 and Table S9 in Supporting Information).

Relationship between gut microbiomes, insect performance, and leaf multi-omics

The relationships between the gut microbiome and the measured variables of leaves were studied using a partial least squares path model (PLS-PM). Insects' feeding performance and the gut microbiome of caterpillars were both correlated with the leaf microbiome and metabolites (Figure 3A). Insects' feeding performance was correlated both directly and indirectly via leaf metabolites with the substrate microbiome (Figure 3A). Leaf nutrients affected caterpillar weight directly (Figure 3A), and further analysis identified nitrite as a potentially important predictor of caterpillar performance (Figure 3B). Characteristic metabolites associated with feeding or the gut microbiome were obtained by machine learning and an analysis of variance. A total of 20 compounds for feeding were identified: 10 metabolites enriched within cabbage leaves growing in the vermiculate system, namely adenine, isoleucine, phenylalanine minor, citric acid, beta-alanine, lysine, tartaric acid, raffinose, oxoproline, and methionine, and 10 other metabolites enriched within cabbage leaves growing in the soil systems, namely lactobionic acid, 4-aminobutyric acid minor, melibiose minor, fructose, ribitol, galactinol, gluconic acid lactone minor, gluconic acid, malate, and glucose (Figure 3C). For the gut microbiome, 15 compounds (Figure 3D), namely, adenosine, phytol, myo-inositol, xylose, adenine, tartaric acid, methionine, beta-alanine, isoleucine, galactinol, melibiose minor, lactobionic acid, glucose, ribitol, and 4-aminobutyric acid were identified. Further, 20 and 21 genera, as microbial signatures of the leaves, were found to be potentially associated with caterpillar feeding performance and the gut microbiome, respectively (Figure 3E and F). Microbes in leaves that were related to caterpillar feeding performance were found to be abundant in the soil systems, while microbes in leaves that were related to the gut microbiome were found to be abundant in the vermiculite system (Figure 3E and F). Several microbial groups present in the soil were related to the gut microbiome, such as *Actinoallomurus*, *Actinospica*, *Amnibacterium*, *Ralstonia*, *Rhizomicrobium*, and *Geobacter* (Figure S4 in Supporting Information); while no soil microbial groups were significantly related to caterpillar feeding performance (Figure S5 in Supporting Information). In the vermiculite system, only one microbial group (*Sediminibacterium*) present in the vermiculite substrate was related to the gut microbiome (Figure S4 in Supporting Information), while vermiculite microbial groups such as *Sphingopyxis*, *Niastella*, *Kinneretia*, *Dyadobacte*, and *Curtobacterium* were related to caterpillar feeding performance (Figure S5 in Supporting Information).

Identifying the effects of leaf metabolites on insects' feeding performance

Different compounds (nitrate and 20 metabolites) with the

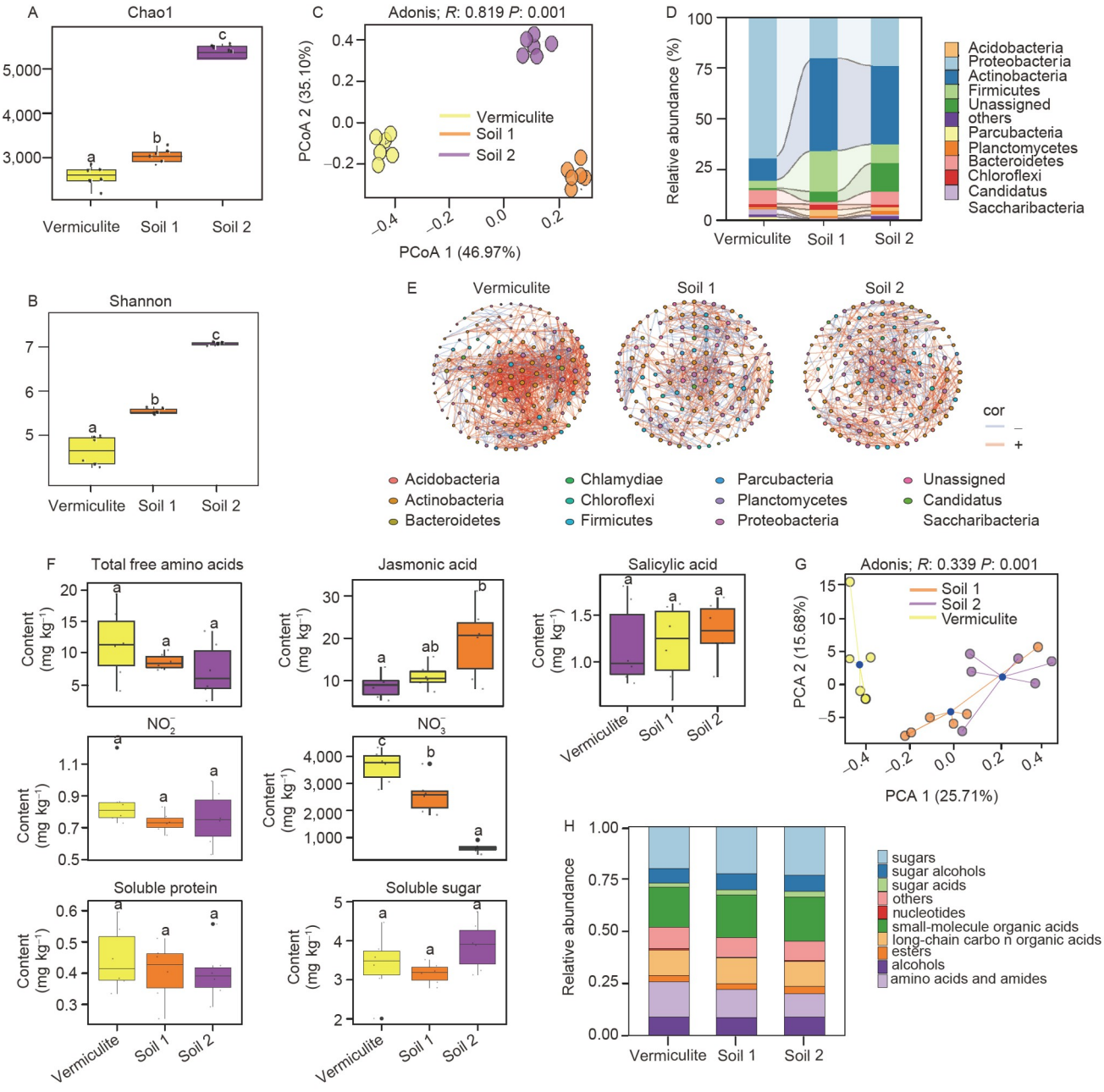

Figure 2 Nutrients, metabolomes and microbiomes of cabbage leaves grown in different systems. A and B, Box plots showing Chao1 and Shannon diversity of leaf bacterial communities. The different letters above bars represent significant differences ($P \leq 0.05$). C, PCoA based on Bray-Curtis dissimilarities performed on the taxonomic profiles at the OTU level. *R*- and *P*-values are based on an Adonis test. D, Relative abundance (%) of the 10 major phyla present in the leaf bacterial communities. E, Co-occurrence networks of the abundant OTUs in the leaf microbiome in the different systems. F, Box plot showing leaf nutrient concentrations in the different systems. G, Principal component analysis (PCA) of the leaf metabolomes in the different systems. *R*- and *P*-values are based on an Adonis test. H, Relative abundance of leaf metabolites grouped according to their chemical properties. Box plots show the median, interquartile range, 1.5× interquartile range, and outliers.

greatest contribution to the separation between soil and vermiculite were selected for the identification of their effects on insects' feeding performance in a bioassay choice experiment (Figure 3B and C). Compounds were mixed-sprayed on the surface of leaves at two concentrations (10 and 50 mmol L^{-1}). In the nitrate test, caterpillars aggregated and fed more from leaves treated with a higher concentration of nitrate (Figure 4A; Figure S6 in Supporting Information). In the test where metabolites were applied at 10 mmol L^{-1}, the insects fed relatively more from leaves with VS metabolites (enriched in a vermiculate system) than from the control leaves and less from leaves with SS metabolites (enriched in a soil system) (Figure 4B). No significant differences were observed when metabolites were applied at the

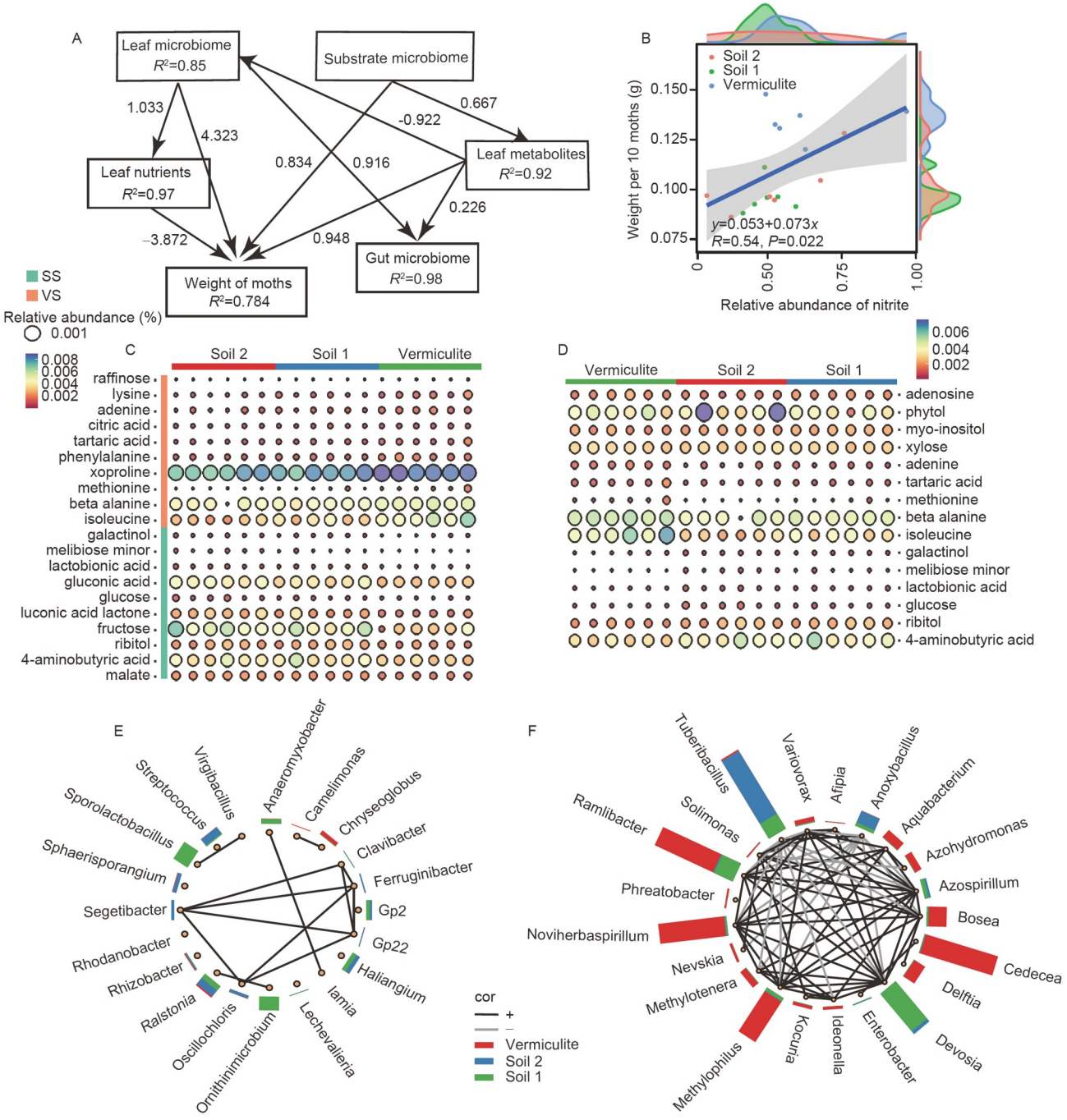

Figure 3 Multiomics correlation analysis among substrate microbiome, plant microbiome, plant metabolome, plant nutritionts and gut microbiome. A, Partial Least Squares Path Modeling (PLSPM). The numbers adjacent to each line in the PLSPM model are the "total effect" values. For each regression in the structural model, we have an R^2 that is interpreted in a similar way to the multiple regression analysis. The R^2 indicates the amount of variance in the endogenous latent variable explained by its independent latent variables. B, The relationship between nitrate salt and larval weight of caterpillars, R is the total variance explained, and P represents significance. C, Bubble plot showing the abundance of 20 metabolites related to caterpillar performance. D, Bubble plot showing the abundance of 15 metabolites related to the gut microbial community. E, Network with stacked bar plots showing the relative abundance of the 20 microbial OTUs of leaves related to caterpillar performance. The length of the bars represents the relative abundance of each genus in the three substrates. The line between nodes represents significant correlations (P<0.05; Mantel test. Positive correlations indicated in black). F, Network showing the relative abundance of 21 microbial OTUs of leaves related to the gut microbial community of the caterpillars; the length of bars represents the relative abundance of each genus in the three substrates. The line between nodes represents significant correlations (P<0.05; Mantel test. Positive correlations indicated in black, negative correlations indicated in gray).

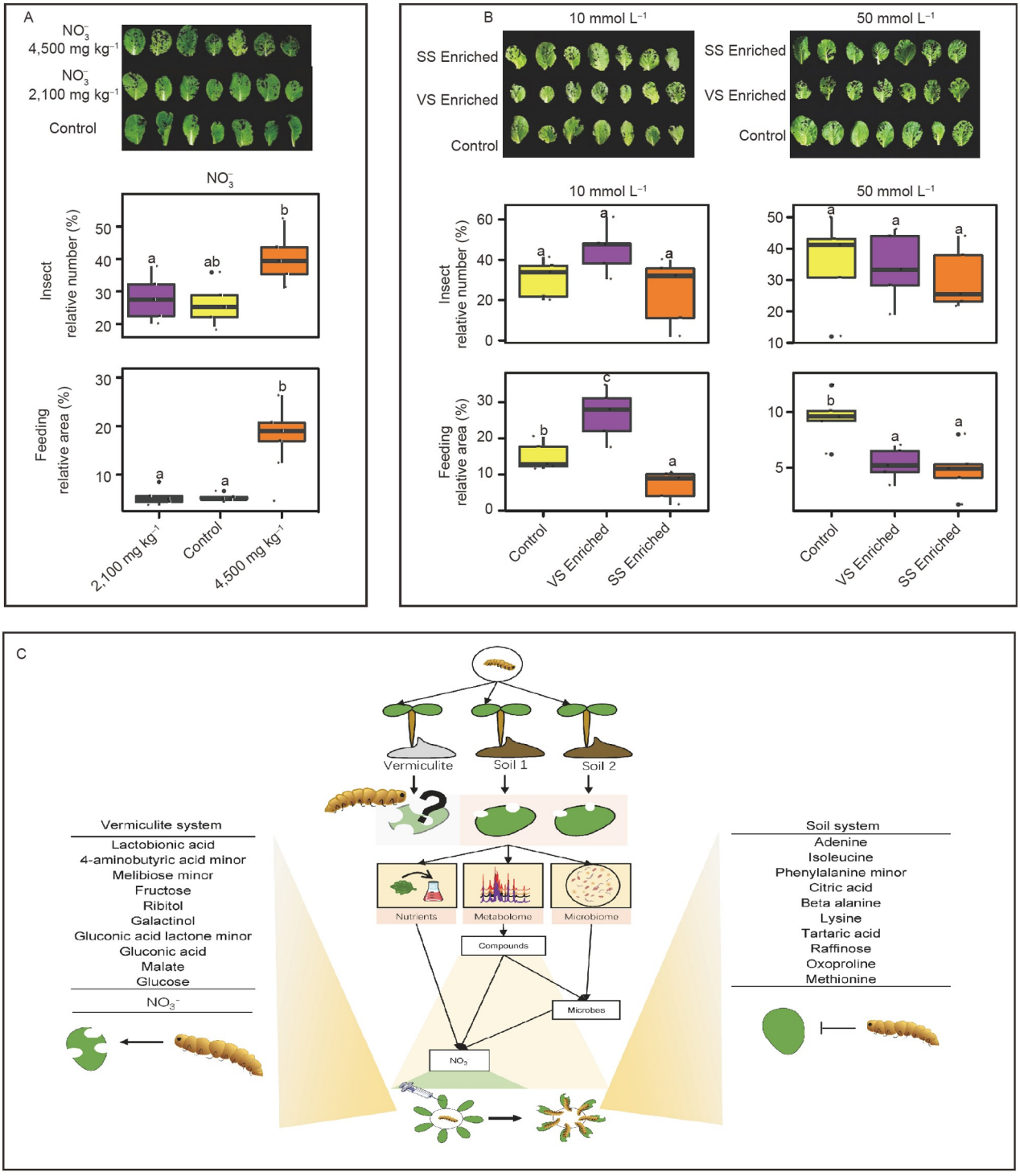

Figure 4 The effect of metabolites on feeding performance. A and B, Bioassay choice experiment with caterpillars feeding on control leaves and leaves treated with two nitrate concentrations (A) and ten compounds enriched in the VS or the SS (B). Each group of compounds was evaluated at two concentrations (10 and 50 mmol L^{-1}). In each replicate, three choices were offered. The ten metabolites enriched within cabbage grown in the soil system included lactobionic acid, 4-aminobutyric acid minor, melibiose minor, fructose, ribitol, galactinol, gluconic acid lactone minor, gluconic acid, malate and glucose. The ten metabolites enriched in cabbage grown in the vermiculate system consisted of adenine, isoleucine, phenylalanine minor, citric acid, beta-alanine, lysine, tartaric acid, raffinose, oxoproline, and methionine. The significant differences between treatments are marked with different letters (Wilcoxon *t*-test, $P<0.05$). C, The conceptual model relates the gut microbiome and feeding to the plant microbiome and metabolome in the different systems.

higher concentration (50 mmol L^{-1}) (Figure 4B; Table S10 in Supporting Information).

DISCUSSION

Our results demonstrate that larvae prefer the leaves of cabbage plants grown in vermiculite substrate to those of plants growing in soil systems. Previous studies have shown that different phytohormone levels, especially SA and JA, can alter herbivore feeding performance. For example, organic soil management has been shown to promote the accumulation of SA in plant tissue, thereby reducing the attractiveness of the crop to pests (Blundell et al., 2020). It has been reported that SA signaling mediated plant defense responses to phloem-feeding insects such as aphids, and JA signaling mediated plant defense responses to leaf-chewing insects such as *Plutella xylostella* (Mewis et al., 2005). In our study, there was no significant correlation between SA content and insect performance across the three substrates (Figure S6 in Supporting Information). However, for JA, there was a trend toward a negative relationship (P=0.056) between JA concentration and insect weight, even though this was consistent only for plants grown in Soil 2 (Figure S7 in Supporting Information). Plant tissue nitrogen is often considered the most important factor determining the performance and feeding of invertebrate herbivores (Mattson, 1980). In the current study, leaves from soilless systems had a higher nitrate content, and these leaves were preferred by insects. Soil fertilizer management regimes have the potential to alter plant tissue metabolites and nutrient content, as plants are expected to be less prone to insect pests and disease incidence when the supply of available nutrients is lower. For situations with high nutrition inputs, such as with the soilless cultivation system in our study, our results suggest the use of more balanced nutrient applications for improved insect pest management.

In plant production, sufficient available nutrient supply in soilless cultivation systems will promote primary metabolism, and plant characteristics in these systems may differ greatly from those grown in soil (SharathKumar et al., 2020; Yang et al., 2018). Plant metabolic characteristics are known to impact the feeding behavior of insects. For example, certain toxic metabolites, such as glucosinolates, are produced by plants to prevent feeding (Ahuja et al., 2011). Interestingly, in our study, changes in other metabolites also influenced the feeding preference of caterpillars. Compared to the soil cultivation system, cabbage grown in soilless substrate not only increased tissue nitrate content but also altered metabolomes. In our study, from the 53 differential expressed compounds (Figure S2 in Supporting Information), the mixture of 10 metabolites that were enriched in the VS stimulated larval feeding in a dose-dependent fashion, while the mixture of 10 metabolites enriched in the soil system resulted in an inhibition of feeding in a dose-independent manner (Figure 4B). In validation experiments, we chose the concentration of 10 and 50 mmol L^{-1} for metabolite solutions, as many of them (e.g., Adenine, 4-aminobutyric acid, tartaric acid, and beta-alanine) had concentrations of less than 30 μmol L^{-1} in plant tissue (Mikulska et al., 1998; Nosarzewski et al., 2012; Shelp et al., 1999; Winter et al., 1992). Our study showed that feeding choices were affected when metabolites were added at concentrations of 10 mmol L^{-1} but not at 50 mmol L^{-1}. The results indicate that the effects of those metabolites enriched in the VS are nonlinear with the concentration; they may recruit insects at lower concentrations while they circumvent at high concentrations. These results exemplify how important growth substrates are for plant-insect interactions and the required pest and disease control in agricultural systems. Depending on the substrate, crops can become more susceptible or more resistant to insect damage. At the same time, the altered metabolome affected the composition of the leaf microbiome and further altered the gut microbiome directly or indirectly via the altered leaf microbiome (Figure 3A).

Animal guts, including those of herbivorous insects, harbor diverse microbiomes that have co-evolved with the host organism. These microbiomes are essential for the health of the host (Bengmark, 1998; Ding et al., 2021; Gill et al., 2006) and can determine, for example, the susceptibility of the organism to diseases (Kimura et al., 2020; Miyauchi et al., 2020; Nemet et al., 2020). In an agricultural context, modification of the gut microbiome of herbivorous insects could potentially be used to enhance crop productivity as it may reduce the damage caused by these herbivores. Alterations in diet can be an effective method to induce changes in the gut microbiome which can occur in as little as one day (Asnicar et al., 2021; Shepherd et al., 2018). Indeed, in the current study, we found that the composition of the microbiome of the leaves and the metabolites present in those leaves were the two key factors that correlated with the feeding performance of the caterpillars and the damage they inflicted on the plant. Similar results were also detected in the PLS-PM analysis, where the weight of *Plutella* was directly correlated with the leaf microbiome and the leaf metabolites. As these characteristics of the leaves were, in turn, influenced strongly by the substrate in which the plants grew, our study shows that decisions about growing substrates can have far-reaching consequences for both the cabbage plants and their natural enemies. Eilers et al. (2016) also reported that insects can cause varying degrees of damage to plants growing in different substrates. Hence, our study suggests substrate choice greatly influences both the microbiome and metabolome of the crop, and these substrate-mediated changes in the crop subsequently influence the feeding performance and microbiome of the natural enemies of the crop.

The gut microbiome of foliar-feeding caterpillars often also contains microbes acquired directly from the substrate/soil where the plants grow (Gomes et al., 2020; Hannula et al., 2019; Kikuchi et al., 2007), and we observed a correlation between the leaf microbiome and the gut microbiome. In our study, 21 bacterial genera from leaves were identified that may affect the gut microbiome, and interestingly, most of these were abundant in the vermiculite cultivation system. We also detected genera present in the soil that correlated significantly with the gut microbiome. Other studies have shown that the soil bacteria *Porphyrobacter* correlated with the growth of both plants and aphids (Zytynska et al., 2020). In our study, these bacteria exhibited a strong association between the soil and gut microbiomes. Similarly, *Geodermatophilus* in the soil was important for the microbiome assembly of ants in another study (Reyes and Cafaro, 2015), and in our study, we found it to be strongly associated with the gut microbiome of caterpillars. Further studies should examine the role of these bacteria in the soil and insect guts in more detail.

Overall, this study illustrates the connectivity of microbiomes in the leaves and insects and the role of crop tissue nutrients and metabolites on the feeding performance of insect pests. Plant nitrate contents and adenine, isoleucine, phenylalanine minor, citric acid, beta-alanine, lysine, tartaric acid, raffinose, oxoproline, and methionine enriched in the soilless cultivation system, all promoted feeding of insects, while lactobionic acid, 4-aminobutyric acid minor, melibiose minor, fructose, ribitol, galactinol, gluconic acid lactone minor, gluconic acid, malate, and glucose enriched in soil cultivation systems decreased feeding (Figure 4C). The composition of both the plant metabolome and plant microbiome explained gut microbiome composition and the feeding behavior of the insects under two cropping patterns. Our study highlights the importance of plant-microbe-insect interactions and the role of the plant metabolome and microbiome on the composition of insect gut microbiomes. Considering that gut microbiomes are increasingly recognized as a major force in affecting insects' feeding performance, further work is required to dissect the particular mechanisms involved, including investigations of how plant metabolomes and microbiomes influence the functioning of the gut microbiome.

MATERIALS AND METHODS

Soil collection

The two soils used in this study were collected from fields with long-term chemical-input management (Soil 1) and organic management (Soil 2) from the Hengxi town of Nanjing, Jiangsu Province (32°02′N, 118°50′E). This region has a typical subtropical monsoon climate, with an average annual temperature of 18°C and annual precipitation of 1,416 mm. The soil is classified as an Ultisol, which is widely distributed throughout the subtropical areas of South China. The soil was collected from two fields, one planted with cabbages and the other with tomatoes. Subsamples of the soil collected from the two fields were stored at −80°C. To both soils, we added 1% organic fertilizer (N 2.2%, P 1.7%, K 1.1%) in the pot experiment. For the vermiculite soilless substrate, we used three substrates in this study. These substrates differed significantly in nutrient availability and soil microbiomes during plant growth (Figures S1 and S8 in Supporting Information).

Greenhouse experiment

Chinese cabbage seed (*Brassica pekinensis*, Cruciferae) was purchased from Hong Kong (Remust) International Co. Ltd. Four seeds were planted in each cell (5 cm×5 cm×5 cm) of seeding trays (50 cells per tray) that were placed in a greenhouse with a 16-h photoperiod (120 μmol photons $m^{-2}s^{-1}$) at 23°C/20°C day/night temperature. After germination, two seedlings in each cell were retained. Three substrates were tested in the experiment; the soils were collected from the two fields and autoclaved vermiculite. Each cell contained 50 g of soil or 10 g of dry vermiculite. For each substrate, we used six trays as six true replicates. Trays were randomly re-positioned in the greenhouse weekly. Each cell was supplied with 2 mL water every morning and evening. In addition, 2 mL of 1/2 MS liquid medium without agar and sucrose (Qingdao Hope Bio-technology Co., Ltd, Qingdao, China) was used instead of water twice a week in the vermiculite medium. After 30 days and before the insects' feeding (see below), leaves from three cells were collected from each replicate tray. The samples from each tray were pooled so that there was one sample per tray and a total of 18 samples (6 replicates×3 substrates). The cabbage leaves were collected by cutting with sterile scissors (by dipping them in 75% ethanol) and then rinsed four times with DNA-free distilled water to remove dust adhering to the leaves. Leaves were then dried with sterile filter paper and stored at −80°C until DNA extraction and phytohormone analysis.

Insect herbivores

Eggs of *Plutella xylostella* (Linnaeus), obtained from Henan Keyun Biological Co. Ltd (Jiyuan, China), were hatched in an incubator at 27°C kept for a 16-h photoperiod (120 μmol photons $m^{-2}s^{-1}$) at 40%–50% relative humidity. After hatching, *Plutella xylostella* (diamondback moth) larvae were placed on the experimental cabbage plants. About 100 larvae were placed in each tray for ten days. Each tray was caged with transparent plastic, and the larvae were allowed to feed on all the plants in the tray. Ten days after introduction, 50 larvae of similar size were harvested from each tray. The larvae were sterilized by dipping them into

75% ethanol for 2 min, followed by rinsing three times with sterilized water. After each replication, all larvae were then transferred to a sterile dish. The guts of the larvae were dissected, placed into a sterile centrifuge tube (1.5 mL) and stored at −80°C until further analyses. Six replicates were collected per substrate.

DNA extraction and amplicon sequencing

From cabbage leaves (after grinding under liquid nitrogen) and insect guts, DNA was extracted using the Power Lyzer PowerSoil DNA Isolation Kit (Qiagen, Germany), according to the manufacturer's protocol. The DNA quality and quantity were assessed through gel electrophoresis and using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Polymerase chain reaction amplification of the V4 region of the bacterial 16S rRNA gene was carried out utilizing the primers 515F: GTGYCAGCMGCCGCGGTAA and 806R: GGACTACNVGGGTWTCTAAT (Walters et al., 2016) which yielded an amplicon length of 292 bp. Amplicon library preparation and high throughput sequencing were performed as described previously (Yuan et al., 2020).

For sequencing data analyses, usearch (Edgar, 2010) (V. 10.1) and vsearch (Rognes et al., 2016) (V. 0.6.3) were used. First, the "vsearch –fastq_mergepairs" script was used to merge paired-end sequences, followed by the "vsearch –fastx_filter" script to remove primer sequences, the "vsearch –derep_fulllength" script to identify unique reads, and the "usearch -cluster_otus" script to generate OTUs at 97% similarity. The OTU tables were created using "vsearch –usearch_global". Taxonomic assignments of OTUs were then performed using the "vsearch –sintax" script and the RDP database (Maidak et al., 2000).

Phytohormone detection

The extraction and analysis of phytohormones (SA and JA) were performed as described by Pan et al. (2008) with minor modifications. Phytohormones were extracted from 200 mg pre-ground fresh leaves in a 2 mL extraction solvent (2:1:0.002, isopropanol:deionized water:hydrochloric acid, v:v:v) and an addition of internal standards (10–50 ng). After vortexing for 30 min at 4°C, 2 mL dichloromethane was added to the suspensions. Samples were vortexed again for 30 min at 4°C, and the suspensions were then centrifuged at 13,000×*g* at 4°C for 5 min. After centrifugation, three phases were formed, and plant debris was placed in the middle of two layers. The lower layer was evaporated with nitrogen evaporators and re-dissolved in 80% methanol. After purification with SPE columns (CNWBOND HC-C18, 200 mg, 3 mL), samples were used for HPLC-MS/MS as described previously (Forcat et al., 2008). The sample was delivered to the ESI source without going through an HPLC column. The delivery solvent consisted of 50% methanol and 50% water at a flow rate of 0.25 mL min^{-1}. The MS was operated at negative ionization and full scan (*m*/*z* 50–1,000) mode. Operational parameters were a capillary voltage of −3.5 kV, fragmentor 135 V, desolvation gas (nitrogen, ≥99.995%) at a flow rate of 10 L min^{-1}, temperature 350°C, and nebulizer (nitrogen, ≥99.995%) pressure 40 psi.

Metabolomes of leaf samples

For the extraction of leaf metabolites, 0.2 g of leaves per sample was finely ground in liquid nitrogen, and the powder was processed as described previously. (D'Alessandro et al., 2013). Mixtures were homogenized in a ball mill for 4 min at 45 Hz, treated with ultrasound for 5 min while incubated in ice water, and centrifuged for 15 min at 14,000×*g* at 4°C. The supernatant (0.75 mL) was transferred into a fresh 2 mL GC/MS glass vial. After evaporation in a vacuum concentrator, 40 μL methoxy amination hydrochloride (20 mg mL^{-1} in pyridine) was added and incubated for 30 min at 80°C, followed by the addition of 50 μL BSTFA (Bis(trimethylsilyl) trifluoroacetamide) reagent (1% TMCS (trimethylchlorosilane), v/v) to the sample aliquots, and incubated for 1.5 h at 70°C. All samples were then analyzed by GC-TOF-MS. GC-TOF-MS analysis was performed using an Agilent 7890 gas chromatograph coupled with a time-of-flight mass spectrometer. The system utilized a DB-5MS capillary column. 1 μL aliquot of sample was injected in splitless mode. Helium was used as the carrier gas, the front inlet purge flow was 3 mL min^{-1}, and the gas flow rate through the column was 1 mL min^{-1}. The initial temperature was kept at 50°C for 1 min, then raised to 310°C at a rate of 10°C min^{-1}, and then kept for 8 min at 310°C. The injection, transfer line, and ion source temperatures were 280°C, 280°C, and 250°C, respectively. The energy was −70 eV in electron impact mode. The mass spectrometry data were acquired in full scan mode with the *m*/*z* range of 50–500 at a rate of 12.5 spectra per second after a solvent delay of 6.27 min (Kind et al., 2009). Raw peak analyses were performed as described by Yuan et al. (2018).

Soluble sugars, proteins, amino acids, nitrate and nitrite in leaves

Leaves from the three substrates (vermiculite, Soil 1, and Soil 2) were harvested and washed with distilled water. Soluble sugars were then determined using the anthracene method (Hansen and Møller, 1975), soluble proteins with the Coomassie bright blue method (He, 2011), free amino acids using the ninhydrin colorimetric method (Chen et al., 2020), and nitrate and nitrite with the spectrophotometric method (Norman and Stucki, 1981) were performed.

Bioassay to test preference of *Plutella xylostella*

A nonparametric test was used to differentiate the leaves' metabolomes between the soil and the vermiculite. The distinct metabolites from Soil 1, Soil 2 and vermiculite were selected (P-value<0.05, with the P-values corrected according to the Benjamini-Hochberg method). Random forest and 10-fold cross-validation were used to identify the top 20 important leaves' metabolites in relative abundance with the greatest contribution to the classification between soil and vermiculite. These metabolites were used to test the preference of *Plutella xylostella*

Cabbage plants were grown for 30 days in one of the substrates, Soil 2, as described previously. The fourth and fifth true leaves were then harvested with sterilized scissors, and the petioles of the leaves were covered with degreased cotton saturated with sterilized water to prevent withering. To determine the effect of different metabolites and nitrates on the feeding preferences of *Plutella xylostella*, a choice experiment was set up. Metabolites or nitrate were sprinkled onto the leaves before the feeding of larvae. Ten metabolites found to be enriched within cabbage leaves growing in the vermiculate system, referred to as VS compounds, were tested: adenine, isoleucine, phenylalanine minor, citric acid, beta-alanine, lysine, tartaric acid, raffinose, oxoproline, and methionine. Ten other metabolites enriched within cabbage leaves growing in the soil systems and referred to as SS compounds were also tested: lactobionic acid, 4-aminobutyric acid minor, melibiose minor, fructose, ribitol, galactinol, gluconic acid lactone minor, gluconic acid, malate, and glucose. Metabolites at equal concentrations for each compound were dissolved in sterilized water at two concentrations (10 and 50 mmol L^{-1}) and sprayed 1.5 mL onto 3 leaves (about 1.5 g per 3 leaves as a replicate); water was used as a control. The solution of nitrate at two concentrations (2.1 and 4.5 g L^{-1}) and water control were sprayed 1.5 mL on 3 leaves (about 1.5 g per 3 leaves as a replicate). KNO_3 was used as nitrate, and the final concentration was calculated based on NO_3^-. This concentration was chosen based on the leaf nitrate content of cabbage grown in the vermiculate and the soil. This resulted in three combinatory settings, and each setting consisted of three treatments: (i) water control, VS compounds and SS compounds at 10 mmol L^{-1}; (ii) water control, VS compounds and SS compounds at 50 mmol L^{-1}; (iii) water control, 2,100 and 4,500 mg kg^{-1} nitrate. For each setting, the three treated leaves were placed at equal distances in one box and 80 *Plutella xylostella* (5-day-old) larvae were placed equidistant from the 3 leaves. The boxes were then sealed with Parafilm. After 17 h, the number of caterpillars on each leaf was recorded, and the area consumed was assessed. For each treatment combination, there were 5 replicates.

Statistical analysis

Microbial community analyses

A normalized number of sequences was randomly extracted from each sample to calculate *alpha* diversity indices that were estimated with the vegan R package (Dixon, 2003). Nonparametric t-tests were used for the detection of significance for the Shannon diversity, Pielou evenness, and Chao1 index in R with the "EasyStat" package. Before the calculation of *beta* diversity, relative abundances were used to standardize the OTU profiles. Bray-Curtis dissimilarity matrices were prepared using the vegan R packages. PERMANOVA (Adonis, transformed data by Bray-Curtis, permutation=999) was used to test if the *beta* diversity differed between treatments. Then, PCoA plots or non-metric multidimensional scaling (NMDS) were generated according to Bray-Curtis dissimilarity matrices created using the R package "ggplot2" We then trained machine learning models by random forest and exhibited the best accuracy rate to predict the classification of samples. The "important" microbes were selected by cross-tabulations in R with "randomForest" Network analysis was performed using the R package "ggClusterNet" (Wen et al., 2022) on GitHub.

Metabolomics analyses

A nonparametric t-test was used to determine significant differences in metabolites and nutrient content between treatments using the "EasyStat" package in R with a false discovery rate (Benjamini-Hochberg). "Unknown" metabolites were filtered out, and a Wilcoxon test was then used to detect the differential abundance of the remaining compounds. P-values were adjusted within the clusters using the Benjamini-Hochberg approach, and metabolites with an adjusted P-value of less than 0.05 were selected. Before the calculation of *beta* diversity, metabolite profiles were standardized to relative abundance and Bray-Curtis dissimilarity matrices were prepared using the vegan R package. PERMANOVA (Adonis, transformed data by Bray-Curtis, permutation=999) was used to determine if *beta* diversity differed significantly between the three substrates, and PCoA plots were generated from Bray-Curtis dissimilarity matrices using the "ggplot" in the R package. Bubble diagrams and heat maps were drawn with the R package "ggplot" and a metabolite network analysis was performed using the R package "ggClusterNet" (Wen et al., 2022). Spearman correlations were used, and a randomly generated network was used as a comparison.

Network analyses

For the networks, the directed graph of the PLS-PM analysis was calculated with the package "plspm" (Russolillo, 2012). A threshold of α=0.05 was considered statistically significant, and P-values were adjusted with a false discovery

rate correction in case of multiple tests (Zeisel et al., 2011). The correlation between metabolome and plant or insect microbiome was estimated using Mantel tests. The R package randomForest was used to construct the random forest model and to test the relationship between the metabolome and the feeding behavior of larvae. A Mantel test was used to correlate the nitrate level and the feeding behavior of larvae and the "ggpubr" R package (https://CRAN.R-project.org/package=ggpubr) was used to produce correlation plots.

Leaf consumption

The consumed leaf area was calculated using ImageJ (Version 1.51k) image analysis software, and a schematic flowchart was drawn using Adobe Illustrator.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. Raw sequence data obtained in this study have been deposited in Genome Sequence Archive in the BIG Data Center, Chinese Academy of Sciences, under accession code CRA004812. All data and code are available on GitHub (https://github.com/taowenmicro/Yuan-et-al.2021).

Compliance and ethics *The authors declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

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