https://doi.org/10.26651/allelo.j/2018-44-2-1164 0971-4693/94 Euro 20.00
Allelopathy Journal 44 (2): 191-200 (July, 2018) *International Allelopathy Foundation* 2018
Table: 1, Figs : 3

Effects of vanillin on cucumber rhizosphere bacterial community

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(Received in revised form: March 25, 2018)

ABSTRACT

Cucumber seedlings grown in soil from a grass field were treated with vanillin at 0.1 μmol/g concentration for 10 days. Its rhizosphere bacterial composition was analyzed by high-throughput sequencing of 16S rRNA genes on an Illumina Miseq platform. A total of 175,273 quality sequences were obtained and these were classified into more than 2,100 OTUs (Operational taxonomic units) at 97% sequence similarity. Principal coordinates analysis at the OTU level, based on both Bray-Curtis and UniFrac distances, showed that vanillin changed the composition of rhizosphere bacterial community in cucumber seedlings. Exogenous vanillin decreased the relative abundances of *Firmicutes* and *Bacteroidetes* phyla but increased the relative abundances of *Actinobacteria*, *Chloroflexi*, *Gemmatimonadetes* and *Cyanobacteria*. At the genus level, exogenous vanillin decreased the relative abundances of *Clostridium sensu stricto 1*, *Lysobacter*, *Ohtaekwangia*, *Devosia* and *Pseudomonas* spp., and increased the relative abundances of *Streptomyces*, *Pedomicrobium* and *Pseudonocardia* spp.

Key words: Allelopathy, bacterial community, cucumber, *Cucumis sativus* L., illumina sequencing, OTU, rhizosphere, vanillin

INTRODUCTION

Allelopathy plays prominent role in plant-plant interactions in both natural and agricultural ecosystems (6,15,40). Allelochemicals can directly exert their toxic effects on plants through interference with plant physiological processes (16,23,49), or indirectly by influencing soil microorganisms and the availability of soil nutrients (7,43,47). After being released into the soil, allelochemicals encounter diverse soil microorganisms, which can modify their persistence, availability and biological activities (7,14). For example, soil microorganisms can metabolize or transform the allelochemicals into other forms with lower or higher phytotoxicity (14). The allelochemicals can also influence the activity, abundance and diversity of soil microbial communities, which may affect the plant performance (13,17,48,52,56). For example, the invasive plant, *Alliaria petiolata* (a European invader of North American forests), suppresses the native plant growth by disrupting the mutual association between the native canopy tree seedlings and the arbuscular mycorrhizal fungi (37).

When a plant specie produces and releases allelochemicals (autotoxins) that can inhibit its own performance is called autotoxicity (36). As an important class of plant secondary metabolites, phenolic compounds have been implicated as autotoxins in several crops [cucumber (*Cucumis sativus*), rice (*Oryza sativa*), tea (*Camellia sinensis*) and

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sugarcane (*Saccharum officinalis*) (17,31,36,52)]. *In-vitro*, phenolic compounds released through the root exudates affects the growth of specific microorganisms viz., *Pseudomonas syringae*, *Fusarium oxysporum*, *Phytophthora drechsleri* and *Rhizoctonia solani* (2,18,42). However, there are few studies to know how these compounds affected the microbial communities in soil.

Earlier we reported that vanillin accumulated in the soil (> 0.08 μmol/g soil concentration) after repeated plantings of cucumber (55). The vanillin inhibited the cucumber seedlings growth and changed the cucumber rhizosphere bacterial community structure as evaluated by PCR-denaturing gradient gel electrophoresis (DGGE) (53). This study aimed to examine the effects of externally added vanillin on cucumber rhizosphere bacterial community composition using the high-throughput amplicon sequencing technique, which can provide a higher taxonomical resolution and a better understanding of environmental microbial communities than the PCR-based fingerprinting techniques (34,41).

MATERIALS AND METHODS

Pot experiment

Cucumber seeds (cv. Jinlv 3), purchased from Tianjin Lvfeng Horticultural High-tech Company, were sterilized in 2.5% sodium hypochlorite and germinated in perlite in growth chamber at 26 °C in dark. Three days later, germinated seeds were planted into plastic pots (10 cm dia, 10 cm depth containing 150 g dry soil and kept in greenhouse (32 °C day/22 °C night, relative humidity : 60-80 %, 16 h light/8 h dark). Each pot contained a single cucumber seedling. The soil used in this experiment was collected from the upper soil layer (0-15 cm) of grass field, Experimental Station, Northeast Agricultural University, Harbin, China (45°41'N, 126°37'E). The soil colour was black (Mollisol), sandy loam texture, organic matter: 3.67%; available N: 89.02 mg kg^{-1}; Olsen P: 63.36 mg kg^{-1}; available K: 119.15 mg kg^{-1}; EC (1:2.5, w/v): 0.33 mS cm^{-1} and pH (1:2.5, w/v): 7.78 (53).

As the phenolic compounds are rapidly metabolized by soil microorganisms after entering the soil, the vanillin was added periodically to the soil as suggested before (51,53). Cucumber seedlings at one-leaf stage were treated 5-times with 0.1 μmol/g soil vanillin every two days. Seedlings treated with distilled water served as control. Each treatment had five seedlings and was replicated thrice with a completely randomized design. The solution pH was adjusted to 7.0 with 0.1 M NaOH solution, because the soil pH is dominant factor to regulate the soil microbial communities (10). Soil water content was maintained at about 60% water holding capacity by maintaining a constant weight of pots.

Rhizosphere Soil Sampling and DNA Extraction

Cucumber rhizosphere soil samples were collected 10-days after the first application of vanillin or water as described before (54). Briefly, cucumber roots were gently removed from pots and the soils loosely attached to cucumber roots were carefully removed by manual shaking. Then, soil attached to root surface was gently brushed off and considered

as rhizosphere soil. After sieving (2 mm), these soil samples were stored at -70°C till analyses. Samples from five plants in each replication of individual treatment were pooled to make a composite sample for each replication. In total, there were six composite rhizosphere soil samples.

Total soil DNA was extracted with the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) as per the manufacturer's instructions. Each composite soil sample was extracted in triplicate and the extracted DNA solutions were pooled. There were six composite DNA samples in total.

High-throughput Amplicon Sequencing and Data Processing

Total soil bacterial community compositions were estimated with high-throughput sequencing on an Illumina MiSeq platform. Primer sets of F338/R806 was used to amplify V3-V4 regions of the bacterial 16S rRNA gene described earlier (8,50). To distinguish each sample, both the forward and reverse primers had a six-bp barcode unique to each soil sample. The PCR protocol was: 95°C for 3 min; followed by 27 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s; and a final extension at 72°C for 10 min. There were three technically replicated PCR reactions for each DNA sample, which are subsequently pooled and purified using an Agarose Gel DNA purification kit (TaKaRa, China). Then, purified amplicons were quantified by a TBS-380 micro fluorometer with Picogreen reagent (Invitrogen, USA), and mixed accordingly to achieve the equal concentration in the final mixture. The mixture was then paired-end sequenced (2×300) on an Illumina Miseq platform at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China.

Raw sequence reads were de-multiplexed, quality-filtered and processed using FLASH (24) as described before (50). Operational taxonomic units (OTUs) were delineated at 97% sequence similarity with USEARCH using an agglomerative clustering algorithm (9). Then, a representative sequence of each OTU was taxonomically classified through BLAST in the SILVA databases (32). Chimeric sequences were identified and removed using USEARCH 6.1 in QIIME (5). To avoid potential bias caused by sequencing depth, a random sub-sampling effort of 25,922 16S rRNA gene sequences per sample was performed for further analysis. The raw sequences of the 16S rRNA deposited in the NCBI-Sequence Read Archive with the submission Accession Number SRP127535.

Statistical Analysis

For llumina Miseq sequencing data, the defined OTUs were used to calculate taxon accumulation curves with the 'vegan' package in 'R' (35). Alpha diversity indices, Chao, ACE, Shannon index and inverse Simpson index were calculated using QIIME (5). For beta diversity analysis, weighed UniFrac distances and Bray-Curtis distances were calculated using QIIME (5) and 'vegan' package in 'R' (35), respectively. Principal coordinates analysis (PCoA) was conducted to visualize the community similarity with the 'vegan' package in 'R' (35). Differences in alpha diversity indices and relative abundances of microbial taxa between treatments were analyzed using Welch's *t* test in 'R' (35).

RESULTS AND DISCUSSION

Illumina Miseq sequencing data

In total, we obtained 175,273 quality bacterial 16S rRNA gene sequences from all samples (mean=29,212). The average read length of these sequences was 397 bp. Across all samples, more than 2,100 OUTs were detected at 97% sequence similarity (data not shown). Rarefaction curves of OTUs of all samples tended to approach the saturation plateau (Figure 1a) and the Good's coverage was larger than 98.5% for each sample (Figure 1b). Therefore, the number of sequences was sufficient to assess the diversity of cucumber rhizosphere bacterial communities.

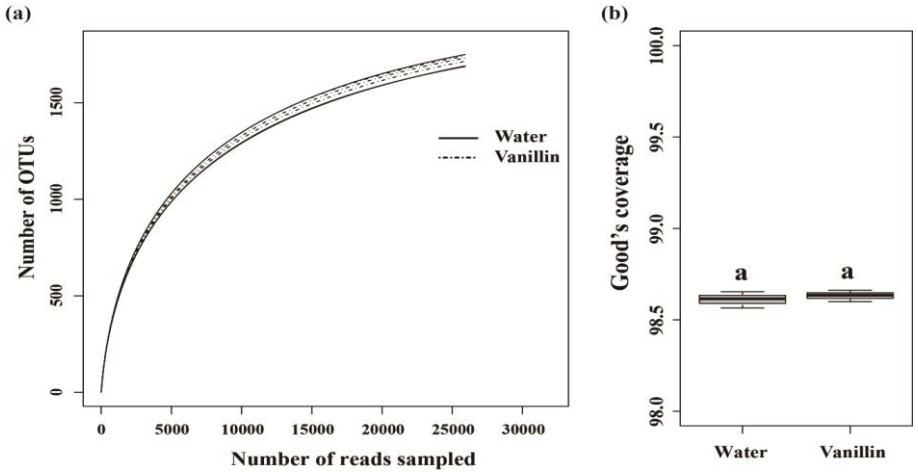

Figure 1. Rarefaction curves of the number of operational taxonomic units (OTUs) (a) and the Good's coverage (b) of cucumber rhizosphere bacterial communities treated with water or vanillin. OTUs were delineated at 97% sequence similarity. Random subsamples of 25,922 16S rRNA gene sequences per sample were used to generate the rarefaction curves and calculate the Good's coverage. Different letters indicate significant difference based on Welch's *t* test ($P<0.05$).

Bacterial community diversity and structure

Cucumber rhizosphere soils treated with water and vanillin had similar bacterial community richness (the number of OTUs observed, ACE and Chao indices) and diversity (Shannon and Inverse Simpson indices) indices (Figure 2a). PCoA analysis at the OTU level, based on both Bray-Curtis and UniFrac distances, showed a clear separation of cucumber rhizosphere soils treated with water and vanillin (Figure 2b).

Taxonomic characteristics of bacterial communities

Taxonomical classification at the phylum level revealed that 31 bacterial phyla were detected in total (data not shown) and 1.39% bacterial sequences were unclassified at this level. The dominant phyla (relative abundance >5%) across all soil samples were *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Firmicutes*, *Chloroflexi* and *Bacteroidetes*, which accounted for more than 92% of the total bacterial sequences (Figure 3a). Compared

with the water treatment (control), the treatment with vanillin had lower relative abundances of *Firmicutes* and *Bacteroidetes* and had higher relative abundances of *Actinobacteria*, *Chloroflexi*, *Gemmatimonadetes* and *Cyanobacteria* ($P < 0.05$).

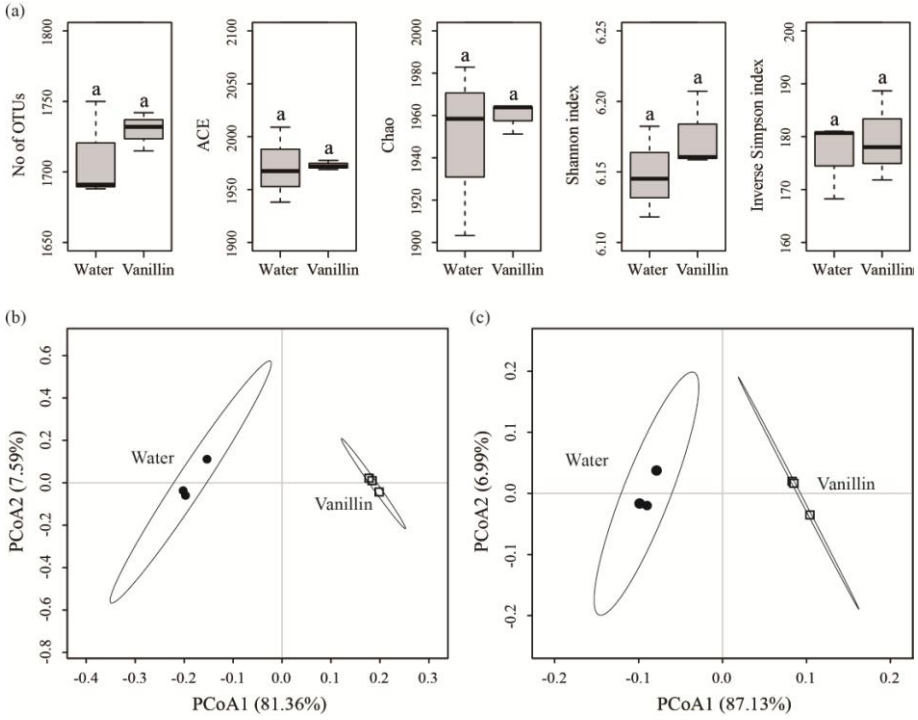

Figure 2. Alpha diversity and beta diversity indices of bacterial communities in cucumber rhizosphere soils treated with water or vanillin.
For alpha diversity, number of OTUs observed (No. of OTUs), ACE, Chao, Shannon and Inverse Simpson indices were calculated using random subsamples of 25,922 16S rRNA gene sequences per sample. OTUs were delineated at 97% sequence similarity. Different letters indicate significant difference based on Welch's *t* test ($P<0.05$). For beta diversity, differences in Bray-Curtis (b) and UniFrac distances (c) of bacterial communities at the OTU level were visualized by principal component analyses. Ellipses indicate 95% confidence interval for replicates.

More than 70 bacterial classes were detected in all soil samples (data not shown). All samples were dominated by *Actinobacteria*, *Acidobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria* and *Clostridia* (relative abundance >5%) (Figure 1b). Compared with control (water treatment), the treatment with vanillin had lower relative abundances of *Clostridia*, *Gammaproteobacteria*, *Cytophagia*, *Erysipelotrichia* and *Sphingobacteriia*, and had higher relative abundances of *Betaproteobacteria*, *Actinobacteria*, *Bacilli*, *KD4-96*, *Gemmatimonadetes*, *Thermomicrobia*, *TK10*, *Cyanobacteria*, *Gitt-GS-136* and *Chloroflexia* ($P < 0.05$).

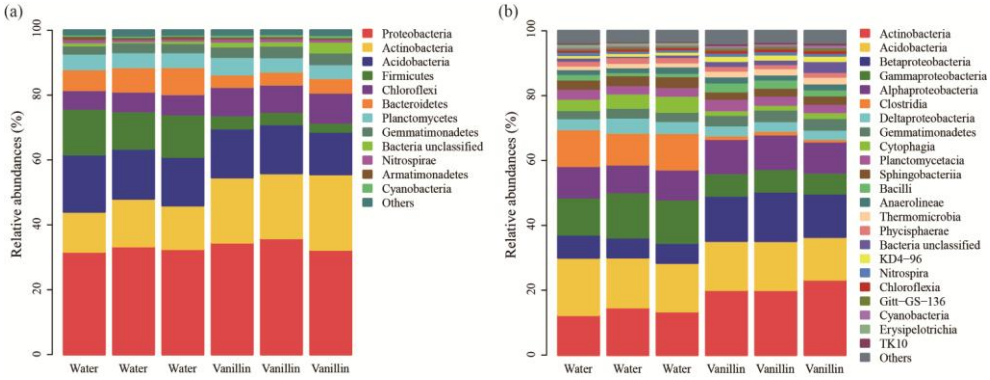

Figure 3. Relative abundances of main bacterial phyla (a) and classes (b) in cucumber rhizosphere soils treated with water or vanillin.
Bacterial phyla and classes with average relative abundances > 0.5% in at least one treatment were shown.

At the genus level, more than 550 bacterial genera or groups were detected (data not shown). Compared with control, the treatment with vanillin had lower relative abundances of *Clostridium sensu stricto 1*, *Steroidobacter*, *Acidibacter*, *Lysobacter*, *Terrisporobacter*, *Bryobacter*, *Turicibacter*, *Ohtaekwangia*, *Dokdonella*, *Ferruginibacter*, *Phenylobacterium*, *Reyranella*, *Devosia*, *Luteimonas* and *Pseudomonas* spp., and had higher relative abundances of *Pseudarthrobacter*, *Skermanella*, *RB41*, *Microvirga*, *Microlunatus*, *Blastococcus*, *Bacillus*, *Streptomyces*, *Pir4 lineage*, *Nordella*, *Pedomicrobium* and *Pseudonocardia* spp. ($P < 0.05$) (Table 1).

Previously (55), we reported that the concentration of vanillin in cucumber rhizosphere was about 0.08 μmol/g soil in a continuous monocropping system. In both natural and agricultural ecosystems, the concentration of soil phenols ranges from 0.01 to 0.5 μmol/g soil (17,21,26,30,55). Therefore, concentration of vanillin used in this study (0.1μmol/g soil) was within the realistic range of concentrations in the soil reported before.

Soil microorganisms play critical roles in terrestrial ecosystems because they have great influences on plant health and fitness, nutrients cycling, and decomposition of organic matter (3,12,25,44). Recently, it is proposed that soil microorganisms can act as targets and mediators of allelopathy (7). As autotoxins of cucumber, phenolic compounds have negative effects on cucumber seedling growth (4,31,46,51,52). In this study, we have further shown the effects of vanillin on cucumber seedling rhizosphere soil bacterial communities. Results showed that exogenously added vanillin changes the cucumber seedling rhizosphere soil bacterial community composition, and vanillin inhibited some bacterial taxa but promoted some other taxa. These results are in conformity with previous studies showing that phenolic acids could act as specific substrates or signaling molecules for some soil microbial species (1) and also inhibit some others (2,18,42).

Crop disease index and soil-borne pathogens usually increases in continuous monocropping systems (20,50,54). In this study, exogenously added vanillin decreased the relative abundances of several plant-beneficial bacterial genera [*Clostridium sensu stricto*

1 (22), *Lysobacter* (11), *Ohtaekwangia* (28), *Devosia* (33) and *Pseudomonas* spp. (45)]. These bacterial genera have plant growth-promoting activities (11,22,28,33,45) viz., producing indole-3-acetic acid, siderophore, and secondary metabolites with antifungal activities. Therefore, the accumulation of vanillin may be linked to the increased plant disease index in monocropping systems by inhibiting the plant-beneficial bacteria. However, this hypothesis should be validated with isolation and *in-vitro* tests of the plant-beneficial characteristics of these genera in the future.

Table 1. Relative abundances (%) of main bacterial genera in cucumber rhizosphere soils treated with water and vanillin.

Bacteria Genus	Water	Vanillin	Bacteria Genus	Water	Vanillin
Clostridium sensu stricto 1	6.21±0.46	0.37±0.06	***Pir4 lineage***	0.33±0.02	0.57±0.04
Panacagrimonas	1.28±0.24	2.06±0.04	*Haliangium*	0.49±0.06	0.37±0.01
Steroidobacter	2.24±0.09	1.09±0.06	*Arenimonas*	0.42±0.00	0.42±0.04
Acidibacter	2.70±0.37	0.56±0.05	*Gemmatimonas*	0.42±0.06	0.41±0.04
Pseudarthrobacter	0.99±0.04	2.14±0.11	*Pseudolabrys*	0.36±0.02	0.41±0.07
Nocardioides	1.40±0.07	1.68±0.08	***Ohtaekwangia***	0.64±0.08	0.13±0.02
Lysobacter	1.78±0.13	0.88±0.03	*Solirubrobacter*	0.31±0.01	0.44±0.04
Terrisporobacter	2.46±0.16	0.19±0.01	*Azohydromonas*	0.22±0.01	0.49±0.08
Gaiella	0.77±0.04	1.86±0.12	*Bradyrhizobium*	0.38±0.03	0.30±0.04
Bryobacter	1.52±0.11	0.91±0.07	*Flavisolibacter*	0.35±0.02	0.32±0.05
Skermanella	0.87±0.09	1.45±0.07	*Ilumatobacter*	0.29±0.02	0.35±0.03
RB41	0.98±0.08	1.32±0.08	***Dokdonella***	0.63±0.09	0.01±0.00
Microvirga	0.64±0.04	1.24±0.06	*Nitrosospira*	0.37±0.00	0.26±0.03
Nitrospira	0.80±0.11	0.92±0.06	***Nordella***	0.21±0.00	0.39±0.02
Microlunatus	0.42±0.06	1.19±0.02	*Gemmata*	0.29±0.05	0.30±0.05
Blastococcus	0.49±0.05	1.09±0.03	*Lapillicoccus*	0.25±0.03	0.32±0.04
Pirellula	0.71±0.08	0.83±0.07	***Pedomicrobium***	0.22±0.03	0.35±0.02
Ramlibacter	0.77±0.02	0.69±0.03	*Dactylosporangium*	0.45±0.11	0.12±0.01
Piscinibacter	0.60±0.02	0.58±0.07	*Massilia*	0.33±0.03	0.22±0.02
Roseiflexus	0.53±0.04	0.63±0.03	***Ferruginibacter***	0.33±0.01	0.21±0.01
Bacillus	0.32±0.07	0.75±0.13	*Opitutus*	0.35±0.08	0.19±0.01
H16	0.46±0.05	0.60±0.05	***Phenylobacterium***	0.35±0.03	0.19±0.02
Chryseolinea	0.89±0.18	0.14±0.02	***Pseudonocardia***	0.16±0.00	0.35±0.02
Marmoricola	0.44±0.02	0.55±0.03	***Reyranella***	0.34±0.03	0.15±0.03
Aeromicrobium	0.50±0.06	0.49±0.05	***Devosia***	0.38±0.03	0.10±0.01
Turicibacter	0.83±0.07	0.13±0.01	***Luteimonas***	0.40±0.03	0.06±0.01
Archangium	0.50±0.01	0.46±0.04	*Aquicella*	0.33±0.09	0.04±0.00
Planctomyces	0.46±0.06	0.48±0.08	*Rhodanobacter*	0.30±0.07	0.01±0.00
Streptomyces	0.35±0.02	0.57±0.04	***Pseudomonas***	0.29±0.03	0.04±0.01

Values (mean±SE) with different letters are significantly different at the 0.05 probability level (Welch's t test), and are highlighted in bold.

Compared with control, the treatment of vanillin had higher relative abundances of *Streptomyces*, *Pedomicrobium* and *Pseudonocardia* spp., which may be involved in degrading the vanillin. *Streptomyces* sp. NL15-2K is capable of degrading the vanillin (27). Similarly some *Pedomicrobium* spp. which possess a multicopper oxidase, are involved in the oxidation of phenolic compounds (38) and *Pseudonocardia* spp. are able to degrade the aromatic compounds (19). It would therefore be promising to isolate and examine these phenolic compound-degrading microorganisms to alleviate the autotoxicity in cucumber.

CONCLUSIONS

Exogenously added vanillin did not significant affect the cucumber rhizosphere bacterial community diversity. However, vanillin changed the cucumber rhizosphere bacterial community composition. Exogenously added vanillin decreased the relative abundances of several plant-beneficial bacterial genera, such as *Lysobacter* and *Pseudomonas* spp. Our results suggested that vanillin may influence the cucumber growth through mediating the plant-rhizosphere microbe interactions.

ACKNOWLEDGMENTS

This work was supported by the University Nursing Program for Young Scholars with Creative Talents in Heilongjiang Province (UNPYSCT-2015002), National Natural Science Foundation of China (31772361), 'Academic Backbone' Project of Northeast Agricultural University (17XG05) and China Agricultural Research System (CARS-23-C-10).

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