A tomato and tall fescue intercropping system controls tomato stem rot

Yunzhuang Zhou a, Huifang Cen a, Danyang Tian a, Chen Wang a and Yunwei Zhang a,b,c

aCollege of Animal Science and Technology, China Agricultural University, Beijing, People’s Republic of China; bNational Energy R&D Center for Biomass (NECB), Beijing, People’s Republic of China; cBeijing Sure Academy of Biosciences, Beijing, People’s Republic of China

ABSTRACT

Intercropping can effectively control some plant soil-borne diseases. However, few studies on intercropping have focused on forage grass as companion plants. In this experiment, Festuca arundinacea (tall fescue) was selected as the intercropping forage to explore whether it could control tomato stem rot. We found that: (1) tomato intercropped with tall fescue had a significantly lower disease incidence and disease index of tomato stem rot than sole tomato; (2) the antifungal activities of the root exudates of tomato and tall fescue in intercropping system were significantly higher than those of sole tomato or tall fescue. Meanwhile, it was inferred that the main allelochemicals might be cyclohexane-1, 2-diol and putrescine based on the GC-MS analysis of root exudates of tall fescue. (3) RNA-seq suggested that intercropping with tall fescue significantly upregulated the expression of genes related to pathogenesis-related proteins and hormone metabolism of tomato compared to those in sole tomato.

INTRODUCTION

Many pesticides are used every year in China (Wang et al. 2017). With the enhancement of awareness of health, people gradually have realized the harm caused potentially by pesticides. Therefore, the continued vigorous development and exploration of effective green biological controls of plant pests and diseases is important. Tomato stem rot is one of the most serious global soil-borne diseases in tomato (Lycopersicon esculentum Mill.) production. The disease symptoms of tomato occurred rapidly after being infected by Rhizoctonia solani Kuhn., which damaged mainly the root and base of the tomato affecting, thereby the fruit yield of tomato (Guo 2015; Wang 2017). The control of tomato stem rot was mostly conducted by using pesticides or changes in cultivation and management measures, which could just control the disease with low efficiency.

All along, reasonable intercropping has been considered a green biological control method that could effectively control plant diseases and insect pests through the rational use of time, space and the interaction between crops (Trenbath 1993; Li et al. 2013). Many studies have shown that superiority and function of the intercropping system were strongly linked with root interactions of different plants, and especially the function of root exudates in intercropping systems was very important to that (Bais et al. 2006; Ren et al. 2008). Intercropping systems have been applied to many plants. However, there are a few of related studies and applications of forage grass in intercropping system. The fact was that some high-quality forage grasses such as tall fescue (Festuca arundinacea Schreb.) and white clover (Trifolium repens L.) had allelopathic effects (Zhu and Shen 2004).

Most tall fescue plants contained endophytic fungi, which built a very stable mutualistic relationship between plants and the fungi (Nan and Li 2004). On the one hand, the photosynthesis products and minerals required for growth of endophytic fungi were provided by the tall fescue. On the other hand, endophytic fungi could produce metabolites such as ergosterol and peramine alkaloids to stimulate the growth and tillering of tall fescue and reduce abiotic stresses (Yang et al. 2015). Rudgers and Clay (2007) found that the endophyte-tall fescue symbiosis could significantly affect the microbial community structure in the soil. Some studies also indicated that endophytic fungi could secrete the 3,11,12-trihydroxycadalene that had inhibitory effects on plant pathogens and could be further developed as biological pesticides (Ma and Zhao 2010). Therefore, the potential exists to use tall fescue as an intercropping plant to control some soil-borne diseases.

The root exudates of tall fescue are not studied before, but it is hard to assume that they might not contain allelochemicals. Furthermore, the study and practices of introducing tall fescue into intercropping systems, especially with vegetables, are very rare. Therefore, this experiment aims to investigate the effects of intercropping tall fescue on the control of tomato stem rot which is a soil-borne disease caused by R. solani. We hope that tomato stem rot could be suppressed in a way of green biological control. Meanwhile, we hope to find more effective intercropping patterns and provide more concepts of intercropping for farmers.

MATERIALS AND METHODS

GROWTH OF PLANTS

The tomato variety ‘Zhongshu 4’ was provided by the Institute of Vegetables and Flowers Chinese Academy of Agricultural Sciences. The tall fescue variety ‘Riding Brand’ was purchased from the Beijing Green Animal Husbandry S&T Development Co., Ltd. All seeds used in this study were surface-sterilized with 1% sodium hypochlorite for 10 min and...
rinsed three times with distilled water before being sown. Seeds of tall fescue were sown into plastic pots (18 cm diameter, 23 cm height) containing an autoclaved (45 min, 121°C) compound medium of soil: vermiculite: humus [1.5:1:1 (v/v/v)] and grown under a greenhouse environment (16/8 h light/dark cycle) in Beijing. After germinating for two days in the petri dish, the tomato seeds were sown in a plug tray and watered (Noorbakhsh and Taheri 2016). 25-days old tomato seedlings (at the 3-true-leaf stage and showing good growth) from the plug tray were transplanted to the middle of a plastic pot (1 seedling/pot), which had been left unplanted for 110 days; other identical tomato seedlings were transplanted into a pot already containing about a hundred 110-days old Festuca seedlings.

**Cultivation of fungi**

The *R. solani* was purchased from the China Centre of Industrial Culture Collection (CICC). The strains were stored at 4°C. The cultivation of mycelium was conducted on PDA medium under an aerobic environment at 27°C with a culture rotation of once every 6 days. In our study, the oat grain was used as a medium for the infection of pathogens to tomato, according to the methods described by Scholten et al. (2001). After being transplanted into each of the plastic pots for 10 days, the stem base of each tomato was inoculated by the oat grains medium with mycelium at the rate of oat grains/soil = 3% (w/w) (Youssef et al. 2016).

**Determination of disease incidence and disease index of tomato stem rot**

The disease incidence and disease index of tomato stem rot were observed and calculated from 9 to 16 days after the inoculation. The pot experiments were conducted with two treatments: tomato intercropped with tall fescue treatment (TF) and sole tomato treatment (ST). Each treatment had 3 replicated blocks and each of them was consisted of 10 pots. The disease incidence was defined as the percentage of tomato seedlings with disease symptoms out of all treated seedlings in each treatment. The disease index was evaluated by a 0–3 scale based on the ratio of disease scab diameter to transverse diameter of the tomato stem (Guo 2015), that was: 0, healthy; 1, disease scab diameter ≤ 1/4 transverse diameter of stem; 2, 1/4transverse diameter of stem < disease scab diameter ≤ 1/2 transverse diameter of stem; 3, 1/2 transverse diameter of stem < disease scab diameter ≤ transverse diameter of stem. The disease index = ∑[(a × b)] / (c × d) × 100 (a was the number of diseased plants at each level; b was the relative disease level; c was the total number of surveys; d was the highest disease level) (Guo et al. 2008). The disease incidence and disease index were evaluated independently with 3 replicated blocks and averaged.

To more intuitively observe the distinguishing status of the different treatments of tomato root structure after the infection of pathogen, a structural scan of the tomato root was performed after 9 days of inoculation. The middle segments with 1 cm length from the different treated tomato roots were picked, soaked in FAA fixative solution for 24 h and submitted to Servicebio Biomart Biotech Co., Ltd. (Wuhan, China) for a vertical cut of the root. Each treatment had three replicates.

**Test of antifungal activities of root exudates in vitro**

Root exudates of tomato and tall fescue were extracted after 9 days of the inoculation according to the methods of Fu et al. (2015). In this study, four treatments were applied: sole tomato treatment (ST), sole tall fescue treatment (SF), tomato intercropped with tall fescue treatment (TF) and tall fescue intercropped with tomato treatment (FT). After that, the root exudates secreted by 1 g fresh weight of root were filtered, freeze-dried and adjusted with sterile deionized water to 5 mL exudate solution (1 g FW-5 mL⁻¹). Then the root exudates were filtered through 0.22 µm Millipore filters and stored at −20°C until further investigation.

The Poison Food Technique was used to determine the antifungal activities of root exudates in vitro (Gao et al. 2014). A total of 600 µL of root exudates of each treatment were added into 5.4 mL of potato dextrose agar (PDA) medium and mixed completely before the solidification. The size of the culture dish was 60 × 10 mm. After the medium was solidified, a hole at 6 mm diameter was made in the center of the medium by a puncher. A mycelia disc at 6 mm diameter cultured for 3 days was placed in the hole and then the new medium was cultured in a dark incubator at 27°C. An equal amount of sterile water was used as a blank control in this study. After three days of culture, the colony diameter in each dish was measured using a ruler in three directions. Each treatment was repeated 4 times. Meanwhile, the root exudates of each treatment were diluted based on the original concentration to form three concentration treatments: 1 g FW.5 mL⁻¹, 1 g FW.10 mL⁻¹ and 1 g FW.20 mL⁻¹.

**Analysis of differently expressed genes (DEGs) in tomato roots by RNA-seq**

After three days of inoculation, total RNA was extracted from the different tomato samples, sole tomato treatment (ST1, ST2, and ST3) and tomato intercropped with tall fescue treatment (TF1, TF2, and TF3), according to the RNAprep pure Plant Kit (TIANGEN, China), respectively. RNA-seq was performed by BIOMARKER TECHNOLOGIES Co., Ltd. (Beijing, China). The purity, concentration and integrity of the RNA samples were performed by Nanodrop, Qubit 2.0 and Agilent 2100, respectively. Each treatment had three replicates.

The concentration of the cDNA library and the insert size were determined using the Qubit 2.0 and Agilent 2100, respectively. Q-PCR was used to accurately quantify the effective concentration of the library. Finally, sequencing was performed using HiSeq X-ten and the sequence read length was PE150.

The sequences of clean reads and reference genomes (ITAG 3.2) were aligned to obtain the position information of each sequence and the characteristic information of the sample by using TopHat2 (Kim et al. 2013). Transcripts and gene expression levels were quantified by using the Cuffquant and Cuffnorm of the Cufflinks software and FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) (Florea et al. 2013). In the quantification process, we used a Pearson correlation coefficient of more than 0.80 as the assessment criteria for consistency among the replicates (Supplementary materials Figure S1); as a
result, we had to abandon one of the sole tomato samples ST3 because of its low Pearson correlation with the other two biological replications. According to the study of Anders and Huber (2010), DESeq was selected for the differential expression analysis among sample groups with a Fold Change (FC) > 2 and a False Discovery Rate (FDR) < 0.01 as the criteria for identification.

**Metabolic analysis of tall fescue root exudates by nontarget GC-MS**

Root exudates of tall fescue intercropped with tomato (FT) and sole tall fescue (SF) stored at −20°C in the previous extraction process of the root exudates (Test of antifungal activities of root exudates in vitro) were sent to BIO-MARKER TECHNOLOGIES Co., Ltd. (Beijing, China) for GC-MS analysis with three replicates. The Spearman Rank Correlation (r) was used as the assessment criteria for consistency among the biological replications of FT and SF (Supplementary materials Figure S2). The specific test conditions were as follows: the analytical instrument for this experiment was an Agilent 7890 GC-TOF-MS equipped with an Agilent DB-5MS capillary column (30 m × 250 μm × 0.25 μm, J&W Scientific, Folsom, CA, USA). The specific analysis conditions of GC-TOF-MS were determined according to the methods reported previously (Kind et al. 2009).

In the experimental process, the P value < 0.05 of the T-test and the Variable Importance for the Projection (VIP) value > 1 of the first principal component of the Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) model were combined as the identification criteria to identify the differential metabolites.

**Statistical analysis**

The original data were conducted by Excel software and SPSS 19.0 software was used for the statistical analysis. Differences between both treatment groups were tested by the independent sample T-test at the P = 0.05 level. Analysis of variance (ANOVA) was performed among the different treated groups, and the means of different treatments were compared by Tukey’s tests at P = 0.05 level. All data were expressed as the mean ± standard error. Spectral data were analyzed for peak extraction, baseline correction, deconvolution, peak integration and peak alignment using the ChromaTOF software (V 4.3x, LECO) during GC-MS analysis.

**Results**

**Effects of intercropping with tall fescue on the disease incidence and disease index of tomato stem rot**

Compared to sole tomato (ST), the disease incidences of tomato intercropped with tall fescue (TF) were decreased by 40.91% and 41.67% at 9 and 16 days after inoculation, respectively (Figure 1(a)). Similarly, the disease indexes of TF also declined significantly (P < 0.05) compared to the ST at 9 and 16 days after inoculation (Figure 1(b)). Meanwhile, based on the results of the disease incidence and disease index at the different infection times, we found that they did not increase with the prolongation of infection time but still maintained a relatively low severity. These results showed that intercropping with tall fescue could effectively control the development of tomato stem rot.

**Effects of intercropping with tall fescue on the cell structure of the tomato root**

To observe visually the effects of intercropping with tall fescue on tomato stem rot, we cut lengthwise and scanned the root of the tomato. The results of scanning, in agreement with the previous results of disease incidence and disease index, indicated that intercropping with tall fescue could effectively alleviate the severity of tomato stem rot. The cortex and parenchyma cells of the sole tomato (ST) were severely deformed and ruptured after 9 days of inoculation (Figure 2(a)). In contrast, the root structure of tomato intercropped with tall fescue (TF) presented a normal performance (Figure 2(b)). Based on a comprehensive analysis of disease incidence, disease index and cell structure of tomato root, we found that a tomato/tall fescue intercropping system could effectively alleviate the development of tomato stem rot and maintain the ability against the disease with the extension of the infection time.

![Figure 1](image-url) Disease incidence and disease index of tomatoes at 9 and 16 days after inoculation: (a) disease incidence of tomatoes after inoculation; (b) disease index of tomatoes after inoculation. ST: sole tomato; TF: tomato intercropped with tall fescue. Different letters on the same day indicated significant differences (P < 0.05).
Figure 2. Root tissues of tomatoes of 9 days after inoculation: (a) sole tomato; (b) tomato intercropped with tall fescue.

Figure 3. Effects of root exudates on the growth of mycelium: (a) root exudates of tomato; (b) root exudates of tall fescue; (c) effects of root exudates on the growth of mycelium (at the concentration of 1 g FW·10 mL$^{-1}$ which means the root exudates secreted by 1 g fresh weight of root were filtered, freeze-dried and adjusted with sterile deionized water to 10 mL exudate solution). CK: sterile water; ST: sole tomato; TF: tomato intercropped with tall fescue; SF: sole tall fescue; FT: tall fescue intercropped with tomato. Different letters on the same day indicated significant differences ($P < 0.05$).
Effects of root exudates on mycelium growth of *R. solani*

In this experiment, root exudates of each treatment group were divided into three concentrations: 1 g FW.5 mL⁻¹, 1 g FW.10 mL⁻¹ and 1 g FW.20 mL⁻¹. Compared to the CK, each concentration treatment of different root exudates had an inhibitory effect on the growth of the mycelium (Figure 3 (a,b)). In addition, the antifungal activities of the root exudates of the tomato and tall fescue in the intercropping system were higher compared to that of the sole tomato and tall fescue in terms of colony diameter (P < 0.05) (Figure 3) except for that of the tomato intercropped with tall fescue (TF) and sole tomato (ST) at the concentration of 1 g FW.20 mL⁻¹. The root exudates of sole and intercropping tall fescue (SF and FT) in different concentrations had significant inhibition effect on the growth of mycelium, indicating that there might be some allelochemicals against the growth of *R. solani*. More importantly, the inhibitory effects of root exudates of tall fescue on mycelium growth did not decrease with the dilution of concentration, which indicated that root exudates of tall fescue had a strong allelopathic effect on the growth of *R. solani*. At the same time, root exudates of ST also inhibited the growth of mycelium compared to the CK, which could suggested that the infection of the pathogen triggered a defence mechanism in the tomato itself.

Analysis of RNA-seq and differentially expressed genes in tomato roots

The total number of clean reads per test sample ranged from 24.21 million to 31.44 million, representing 92.87% to 93.96% of the total readings, which were assessed as the high quality sequences for further analysis (Supplementary materials Table S1). The sequence of each sample was aligned with the reference genome of *Solanum lycopersicum* (ITAG 3.2) (Table 1). The results of the DEseq analysis showed a total 512 upregulated differentially expressed genes (DEGs) in TF compared to ST (Figure 4). To confirm the reliability of the RNA-seq results, 12 genes were randomly selected for qRT-PCR quantification, including 8 upregulated genes, 2 downregulated genes and 2 genes with almost no difference in expression patterns, the primers of which were designed (Supplementary materials Table S2). Actin gene of tomato was the internal reference gene (Forward primer: GAAA-TAGCATAGATGGCAGACG; Reverse primer: ATACC-CACCACCAACAGAT). QRT-PCR results showed the consistency with RNA-seq data (Supplementary materials Figure S3), which indicated the Illumina sequencing in our study was highly reliable.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used to determine the functions and pathways of all differentially expressed genes of tomato (Figures 5 and 6). Cells, cell fractions, organelles and membrane fractions were the most enriched GO terms induced by intercropping with tall fescue in the cellular component group. In addition, metabolic processes, cellular processes, single-tissue processes and response stimuli were the main enriched GO terms of the biological process group and all of them were involved in response to pathogen infections. All enriched molecular functional classes were involved in the metabolic and transcriptional regulation of tomato roots infected with *R. solani*. In the analysis of annotated KEGG pathways, the plant hormones signal transduction pathway and phenylpropanoid biosynthesis pathway were the most enriched pathways, each of which containing 35 DEGs followed by the phenylalanine metabolism pathway (29, 8.15%), biosynthesis of amino acids and carbon metabolism pathway (28, 7.87%), plant-pathogen interaction pathway (26, 7.30%), protein processing in endoplasmic reticulum (21, 5.90%) and glutathione metabolism pathway (19, 5.34%). The top 20 enriched KEGG pathways were shown in Table 2.

According to the different ways of action to resist tomato stem rot, we found some DEGs which had a significantly upregulated expression in the roots of tomato intercropped with tall fescue (Table 3), such as genes encoding branched-chain amino acid transaminase related to disease defence (Solyc12g088220.2, 24.6 times higher in the TF than ST), chalcone synthase related to stimulus and stress (Solyc05g053170.3, 50.6 times), myb-related protein (Solyc02g089190.2, 17.9 times) associated with signaling transduction and ethylene-responsive transcription factor related to plant hormone metabolism (Solyc06g035700.1, 13.5 times). In addition to ethylene, the genes involved in salicylic acid signaling (like TGA), WRKY transcription factor and signal transduction of jasmonic acid were all significantly upregulated in TF. Most of these up-regulated genes have been reported to be associated with disease-resistant in plant. Meanwhile, based on these genes, we can also realize that the resistance to pathogens in plant is very complicated.

Metabolic analysis of tall fescue root exudates by nontarget GC-MS

For the analysis of volatile components of tall fescue root exudates, a principal components analysis (PCA) model about effective was established. In the PCA score plot, SF and FT occurred in significantly different areas, respectively; SF was on the right side and FT was on the left side, which indicated a significant difference in the first principal component between the two samples (Figure 6(a)). Meanwhile, the OPLS-DA model was used to present clearly the correlation of the principal components among samples by using

### Table 1. Alignment statistics result with reference genome for different samples of tomato.

| Sample | Total Reads | Mapped Reads | Uniq Mapped Reads | Multiple Map Reads |
|--------|-------------|--------------|-------------------|--------------------|
| ST1    | 6284678     | 53743770(85.46)%* | 52916236(84.15)%* | 827534(1.32)%*     |
| ST2    | 48421678    | 39763640(82.12%)  | 39167460(80.89%)  | 596180(1.23%)      |
| ST3    | 51173706    | 40666749(79.47%)  | 40139569(78.44%)  | 527800(1.03%)      |
| TF1    | 53562108    | 46399793(86.63%)  | 45911507(85.72%)  | 488286(0.91%)      |
| TF2    | 51550106    | 43887668(85.14%)  | 43423572(84.24%)  | 464906(0.90%)      |
| TF3    | 51130786    | 42591419(83.30%)  | 42135331(82.41%)  | 456088(0.89%)      |

*Note: ST: sole tomato; TF: tomato intercropped with tall fescue; reference genome: ITAG 3.2
*Indicates percentages in total reads.
SIMCA software. The R (3.3.2) package (ropls) was used to evaluate the OPLS-DA model. The evaluation parameters of the model were R2X, R2Y and Q2, where R2X, R2Y and Q2 represented the interpretation rates of the model for the X, Y matrices and the prediction ability of the model, respectively. The closer these three indicators were to 1, the more stable and reliable the model was. The model was considered valid when Q2 > 0.5 and excellent model when Q2 > 0.9. The current OPLS-DA model could be reliably used as a valid model to explain the metabolic differences between the two groups based on the main quality parameters (Q2 = 0.711 > 0.5, R2X = 0.573, R2Y = 0.993) and no dots crossed the corresponding line except a red one in the OPLS-DA model verification diagram (Figure 6(b,c)).

Differentially expressed metabolites were identified based on the VIP value (threshold > 1) of the first principal component in the OPLS-DA model combined with the P value (threshold P < 0.05) of a one-dimensional statistical T-test. The spot size was proportional to the level of difference in the metabolites. A total of 38 significantly upregulated metabolites (P < 0.05) were detected in the root exudates of FT (Figure 7). 15 significantly upregulated metabolites could be identified, which mainly included organic acids, amides and alcohols (Table 4). In particular, the contents of cyclohexane-1, 2-diol and putrescine in the root exudates of FT were 7.08 times and 3.58 times as high as that of SF, respectively, which might be the main allelochemicals in this study.

## Discussion

Intercropping has been widely reported in the literature and commercial implementation about alleviating plant disease via enriching the species of field crops (Ratnadass et al. 2012). Our results showed that intercropping with tall fescue could alleviate the development of tomato stem rot based on the disease incidence and disease index (Figure 1). As a non-host plant of *R. solani* Kuhn., tall fescue could dilute the number of pathogens for host plant tomato. More importantly, due to the dense fibrous root system of tall fescue, the root system of each tomato plants intercropped with tall fescue in the pot experiment was in full contact with the root system of the tall fescue. The dense roots of the tall fescue could form a ‘root wall’ that slowed down the spread speed and intensity of the pathogen infecting the tomato roots to physically control tomato stem rot. In addition, the disease incidence and disease index of tomato intercropped with tall fescue in this experiment were not further aggravated with the prolongation of the infestation time, which showed that the effect of intercropping on controlling plant disease was relatively stable and did not weaken over time.

Many studies have shown that some nonhost plants could secrete allelochemicals into the rhizosphere to help the host plant resist plant pathogens (Bais et al. 2006; Hao et al. 2010; Gao et al. 2014). In this study, the root exudates of
sole and intercropping tall fescue could significantly inhibit the growth of mycelium of \textit{R. solani}. Moreover, the inhibitory intensities of the root exudates of sole and intercropped tall fescue on mycelium growth were not alleviated with the dilution of the concentration, which indicated that the root exudates of tall fescue contained some potent allelochemicals against the growth of \textit{R. solani}. In addition, Nan and Li (2004) reported that the endogenous fungi of forage grass could secret secondary metabolites to improve the resistance of the host plant to the diseases caused by the microbial sources and insect. In this experiment, tall fescue was a known forage grass containing endophytic fungi, therefore, the allelochemicals against \textit{R. solani} might also originate from its own endophytic fungi. At the same time, we found that the inhibition effects on \textit{R. solani} by root exudates of intercropped tall fescue were more strong than that of sole tall fescue (Figure 3 (b)), indicating that intercropping with tomato inoculated with pathogens stimulated the expression and secretion of allelochemicals in the root of tall fescue. Gao et al. (2014) also obtained the same result: root exudates of intercropping

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{GO.png}
\caption{GO (Gene Ontology) functional classification of differentially expressed genes of roots between sole tomato and tomato intercropped with tall fescue.}
\end{figure}
corn were more effective to inhibit the growth of pathogens than that of sole maize. More importantly, we found that the root exudates of intercropped tomato, as a host plant of R. solani, showed a significantly inhibitory effect on the growth of the mycelium (Figure 3(a,c)). This result showed that the resistance of root exudates of intercropped tomato was significantly improved. This was similar to the study reported by Fu et al. (2015), which demonstrated that the root exudates of tomato intercropped with potato onion (Allium cepa L., Aggregatum group) had a significant inhibitory effect on the mycelium growth of pathogens. These results suggested that the mechanism of resistance to disease by intercropping might not be initiated by just one plant or conducted by a certain allelochemicals of companion plants. In our study, the root exudates of tall fescue itself contained allelochemicals to inhibit the growth of pathogen. At the same time, the introduction of tall fescue and pathogens into the intercropping system also induced the improvement of the disease resistance of the host plant tomato. These factors made corporately the intercropping system alleviate the same time, the introduction of tall fescue and pathogens into the intercropping system also induced the improvement of the disease resistance of the host plant tomato. These factors made corporately the intercropping system alleviate the tomato stem rot. As demonstrated by Hage-Ahmed et al. (2013), root exudates of tomatoes could achieve inhibition on the spore germination of Fusarium oxysporum f. sp. lycopersici only when tomatoes were inoculated simultaneously with arbuscular mycorrhizal fungi (AMF) and Fusarium oxysporum f. sp. Lycopersici rather than inoculated with AMF or Fol alone. Therefore, further exploration is needed on the mechanism of the improved resistance to disease of host plant tomato intercropped with tall fescue and the role of pathogen in this process.

**Table 2.** Top 20 enriched KEGG pathways by differentially expressed genes of roots of sole tomato and tomato intercropped with tall fescue.

| Number | Pathway                                                                 | DEGs with pathway annotation | Pathway ID |
|--------|-------------------------------------------------------------------------|-------------------------------|------------|
| 1      | Plant hormone signal transduction                                        | 35(0.83%)                     | ko00750    |
| 2      | Phenylpropanoid biosynthesis                                             | 35(0.83%)                     | ko00940    |
| 3      | Phenylalanine metabolism                                                | 29(0.85%)                     | ko00360    |
| 4      | Biosynthesis of amino acids                                             | 28(0.87%)                     | ko01230    |
| 5      | Carbon metabolism                                                       | 28(0.87%)                     | ko01200    |
| 6      | Plant-pathogen interaction                                              | 26(0.73%)                     | ko04626    |
| 7      | Protein processing in endoplasmic reticulum                             | 21(0.59%)                     | ko04141    |
| 8      | Glutathione metabolism                                                  | 19(0.54%)                     | ko00480    |
| 9      | Amino sugar and nucleotide metabolism                                   | 15(0.41%)                     | ko00520    |
| 10     | Citrate cycle (TCA cycle)                                               | 13(0.35%)                     | ko00020    |
| 11     | Valine, leucine and isoleucine degradation                               | 13(0.35%)                     | ko00280    |
| 12     | Glycolysis / Gluconeogenesis                                             | 13(0.35%)                     | ko00010    |
| 13     | Starch and sucrose metabolism                                           | 13(0.35%)                     | ko00500    |
| 14     | Fatty acid metabolism                                                   | 11(0.30%)                     | ko01212    |
| 15     | Flavonoid biosynthesis                                                  | 10(0.28%)                     | ko00941    |
| 16     | Phenylalanine, tyrosine and tryptophan biosynthesis                      | 10(0.28%)                     | ko00400    |
| 17     | Cysteine and methionine metabolism                                       | 10(0.28%)                     | ko00270    |
| 18     | Stillbenoid / dihydroxyphenanoid and gingerol biosynthesis               | 9(0.23%)                      | ko00945    |
| 19     | Terpenoid backbone biosynthesis                                         | 8(0.25%)                      | ko00900    |
| 20     | Pyruvate metabolism                                                     | 8(0.25%)                      | ko00620    |

*Indicates percentages in total 356 differentially expressed genes. DEGs: differentially expressed genes; KEGG: Kyoto Encyclopedia of Genes and Genomes.

**Table 3.** Significantly upregulated expression patterns of differentially expressed genes (DEGs) of roots of tomato intercropped with tall fescue.

| Gene ID                      | FDR   | Log2FC | Description                                |
|------------------------------|-------|--------|--------------------------------------------|
| Solyc12g088220.2             | 0.00315234 | 4.68  | branched-chain amino acid aminotransfer     |
| Solyc03g033710.3             | 0.00012033 | 2.41  | peroxidase 72                               |
| Solyc02g090380.3             | 0.00000804 | 1.64  | probable disease resistance protein A5g66900 |
| Solyc02g083760.3             | 0.00098548 | 1.44  | pathogenesis-related protein 5              |
| Solyc05g056550.3             | 0.00291063 | 1.12  | serine/threonine-protein kinase SAPK1-like   |
| Solyc05g053130.3             | 0.00008818 | 5.69  | chalcone synthase                           |
| Solyc10g085350.2             | 0.00862842 | 4.9   | phenylpyruvate tautomerase                  |
| Solyc12g011300.2             | 0.00000385 | 4.44  | probable glutathione 5-transferase          |
| Solyc11g072310.2             | 0.00691905 | 2.67  | gibberellin-20-oxidase                      |
| Solyc05g050870.3             | 0.00000053 | 2.86  | cationic peroxidase 1-like                  |
| Solyc06g035700.1             | 0.00834741 | 3.76  | ethylene-responsive transcription factor ERF025-like |
| Solyc06g075510.3             | 0.00634741 | 2.27  | AP2 transcription factor SIAP2e             |
| Solyc12g017370.2             | 0.00120933 | 1.33  | myb family transcription factor APL isoform X1 |
| Solyc08g065190.3             | 0.00182163 | 1.27  | zinc transporter 4, chloroplastic            |
| Solyc12g014610.2             | 0.00720797 | 1.02  | probable WRKY transcription factor 20 isoform X1 |

**Table 4.** Analysis of PCA (principal components analysis) and OPLS-DA (Orthogonal Partial Least Squares Discriminant Analysis) of two groups of tall fescue root exudates: (a) Score plot of the PCA model obtained for SF and FT; (b) Model verification diagram of OPLS-DA; (c) Score plot of OPLS-DA model obtained for SF and FT. DG = SF: sole tall fescue; JG = FT: tall fescue intercropped with tomato.
Some studies confirmed that the increasing disease resistance of the host plant in intercropping system was related to the enhancement of expression of the disease resistance-related genes (Schmid et al. 2013; Fu et al. 2015). In this experiment, the expression of genes involved in disease resistance was enhanced in roots of tomato intercropped with tall fescue (Table 3). By further analysis of the KEGG pathway, we found that expressions of genes involved in signal transduction and metabolism of some hormones and secondary metabolites in roots of tomato intercropped with tall fescue were enhanced, which might increase antifungal activity of root exudates of tomato intercropped with tall fescue. At the same time, the expression level of the genes involved in response to external stimuli and stress in roots of intercropped tomato was higher than that of the sole tomato, especially the gene encoding chalcone synthase which was a key enzyme involved in flavonoid synthesis and genes involved in diterpenoids synthesis. Some studies found that flavonoids and diterpenoids were substances with allelopathic effects and played

![Figure 7. Volcano diagram of differentially expressed metabolite of root exudates between sole tall fescue and tall fescue intercropped with tomato. VIP: Variable Importance for the Projection.](image)

**Table 4. Qualitative differentially expressed metabolites between the root exudates of sole tall fescue and tall fescue intercropped with tomato.**

| Number | Analyte                           | Mass | Similarity | Retention time (min) | Fold change | P value   | VIP value |
|--------|----------------------------------|------|------------|----------------------|-------------|-----------|-----------|
| 1      | cyclohexane-1, 2-diol            | 69   | 454        | 10.28                | 7.07(up)    | 0.0298    | 1.484     |
| 2      | putrescine                       | 174  | 667        | 16.21                | 3.58(up)    | 0.0058    | 1.576     |
| 3      | 2-Deoxyuridine                   | 155  | 488.5      | 10.32                | 2.71(up)    | 0.0327    | 1.417     |
| 4      | trans-3, 5-Dimethoxy-4-hydroxycinnamaldehyde | 174  | 459.5      | 19.13                | 2.55(up)    | 0.0248    | 1.485     |
| 5      | 4-hydroxybutyrate                | 147  | 919.5      | 9.93                 | 2.31(up)    | 0.0430    | 1.445     |
| 6      | 3-hydroxybutyric acid            | 177  | 467.6      | 8.64                 | 2.13(up)    | 0.0142    | 1.474     |
| 7      | epsilon-Caprolactam              | 131  | 225.3      | 10.58                | 1.92(up)    | 0.0273    | 1.430     |
| 8      | aniline-o-sulfonic acid          | 303  | 309.7      | 15.98                | 1.86(up)    | 0.0042    | 1.523     |
| 9      | malonamide                       | 350  | 350.6      | 12.82                | 1.83(up)    | 0.0315    | 1.344     |
| 10     | ribose                           | 103  | 864.5      | 15.48                | 1.83(up)    | 0.0420    | 1.369     |
| 11     | caprylic acid                    | 201  | 933        | 10.36                | 1.64(up)    | 0.0257    | 1.484     |
| 12     | 4-Hydroxyphenyl Ethanol          | 179  | 584.8      | 14.35                | 1.57(up)    | 0.0469    | 1.371     |
| 13     | 3-Methyloxindole                 | 219  | 475        | 14.03                | 1.53(up)    | 0.0305    | 1.410     |
| 14     | ribulose-5-phosphate             | 116  | 331.3      | 19.69                | 1.49(up)    | 0.0316    | 1.431     |
| 15     | pelargonic acid                  | 215  | 951.5      | 11.68                | 1.41(up)    | 0.0054    | 1.550     |
| 16     | succinic acid                    | 188  | 614.6      | 11.02                | −1.24(down) | 0.0337    | 1.424     |
| 17     | gluconic acid                    | 333  | 596.6      | 18.97                | −1.79(down) | 0.0106    | 1.579     |
| 18     | galactinol                       | 204  | 877.1      | 26.71                | −2.23(down) | 0.0279    | 1.515     |
| 19     | dehydroshikimic acid             | 134  | 570.8      | 16.78                | −2.725(down) | 0.0247    | 1.538     |

Note: VIP: Variable Importance for the Projection.
important roles in the plant resistance to adverse environmental stresses (Xie et al. 2011). In addition, in our study, the expressions of genes involved in response to ethylene signaling, salicylic acid signaling (such as TGA) and jasmonic acid signal transduction were significantly upregulated in intercropped tomato. These results indicated that plant hormones might play important roles in the defence against R. solani in tomato, which was consistent with the results of studies by Chen et al. (2003). Therefore, we thought that intercropping with tall fescue enhanced the expression of genes related to tomato disease resistance, which further increased the antifungal activity of tomato root exudates and then alleviated development of tomato stem rot.

In our study, the root exudates of tall fescue exhibited inhibitory effects on the growth of R. solani and alleviated the development of tomato stem rot, indicating that there might be some allelochemicals which were not beneficial to the growth of R. solani. Based on the hypothesis, we carried out a non-targeted metabolic comparative analysis for main components of root exudates between sole and intercropped tall fescue by using GC-MS analysis. For 15 metabolites with upregulated expression in the root exudates of tall fescue intercropped with tomato than sole tall fescue, they could be mostly grouped as an organic acids group like hydroxybutyric acid; amides group like epsilon-caprolactam; and alcohols group like cyclohexane-1, 2-diol (Table 3). In particular, the content of cyclohexane-1, 2-diol was the most significantly improved among these metabolites. This substance might be a potential allelochemical in the root exudates of tall fescue. The significantly upregulated expressions of amides and organic acids in the roots of tall fescue intercropped with inoculated tomato by R. solani were consistent with the results of many studies: amides and organic acids were main secretions with certain allelopathic effects under environmental stress (Yu et al. 2013; Yu et al. 2013). At the same time, the large accumulation of organic acids (such as hydroxybutyric acid, pelargonic acid and caprylic acid) in this experiment would also reduce the pH of the soil environment affecting the growth of R. solani around the tomato (Han 2000). In addition, the content of putrescine in the root exudates of intercropped tall fescue was 2.58 times higher than that of sole tall fescue. Therefore, according to the current experimental results, the main allelochemicals in the root exudates of tall fescue were presumed to be cyclohexane-1, 2-diol, putrescine, organic acids and amides. Notably, the plant root exudates were complex mixtures and widespread synergy effects existed among the allelochemicals.

**Conclusion**

Intercropping with tall fescue not only could significantly alleviate the development of tomato stem rot but also were stable and persistent. The antifungal activities of root exudates of tomato and tall fescue in intercropping system were significantly higher than that of sole tomato and tall fescue. The GC-MS analysis of root exudates of tall fescue showed that the main allelochemicals that inhibited the growth of R. solani might be cyclohexane-1, 2-diol, putrescine, organic acids and amides, especially the content of cyclohexane-1, 2-diol, which was 6.08 times higher in root exudates of intercropping tall fescue than that in sole tall fescue. The intrinsic mechanism of enhanced resistance in intercropped tomato was preliminarily explained by the upregulated expression of genes related to disease resistance in tomato, which further increased the antifungal activity of tomato root exudates and then alleviated development of tomato stem rot.

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**Notes on contributors**

Yunwei Zhang is professor at China Agricultural University in the College of Grassland Science and Technology. Her research is focusing on grass plant breeding, especially on improving the quality and resistance to environmental stress of forage and bioenergy grass (Switchgrass) by elucidating the mechanism of physiological and molecular. Yunzhuang Zhou and Chen Wang are master of Yunwei Zhang at China Agricultural University in the College of Grassland Science and Technology. Their research is focusing on the effects of intercropping on some pests and diseases. Hufang Cen and Danyang Tian are PhD of Yunwei Zhang at China Agricultural University in the College of Grassland Science and Technology. Their research is focusing on improving the quality and resistance to environmental stress of forage and bioenergy grass.

**ORCID**

Yunzhuang Zhou http://orcid.org/0000-0003-1422-6302

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