Exogenously applied ferulic acid and \( p \)-coumaric acid differentially affect cucumber rhizosphere *Trichoderma* spp. community structure and abundance

**Muhammad Khashi U Rahman\(^{1,2}\), Shengcheng Tan\(^{1,2}\), Changli Ma\(^{1,2}\), Fengzhi Wu\(^{1,2}\), Xingang Zhou\(^{1,2}\)**

\(^1\)Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Northeast Region), Ministry of Agriculture and Rural Development, Northeast Agricultural University, Harbin, P.R. China

\(^2\)College of Horticulture and Landscape Architecture, Northeast Agricultural University, Harbin, P.R. China

*Corresponding authors: xingangzhou@yahoo.com; fzwu2006@aliyun.com


**Abstract:** Continuous monocropping can cause the buildup of autotoxins (e.g., phenolic compounds) in the soil, which can alter soil microbial community and inhibit plant growth. However, how different phenolic compounds affect certain soil microbiota is unclear. Here, we studied the response of cucumber rhizosphere *Trichoderma* spp. community to exogenously applied ferulic and \( p \)-coumaric acids by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and real-time PCR techniques. Results showed that ferulic acid, but not \( p \)-coumaric acid, increased the *Trichoderma* spp. abundance, and this increase were positively correlated with ferulic acid concentration. Moreover, ferulic acid changed the community structure, increased the number of DGGE bands, Shannon wiener, and evenness index values, while \( p \)-coumaric acid had no effect on all these parameters of *Trichoderma* spp. community. These results suggest that these two phenolic acids affected *Trichoderma* spp. differentially at the community level.

**Keywords:** Cucumis sativus L.; microorganism; root exudate; antioxidant; soil sickness; allelochemicals

Soil is a complex system comprising a variety of microorganisms functioning by interacting with each other and with the environment (Torsvik and Øvreås 2002). These microorganisms are major components of several plant-soil processes and indicators of plant productivity (Tilak et al. 2005). The functioning of the rhizosphere microbial communities is highly affected by land management practice, i.e., cropping systems (Hamid et al. 2017, Zhou et al. 2017, Hussain et al. 2018, Jin et al. 2020a). The continuous monocropping system usually results in the build-up of plant autotoxins, soil-borne pathogen, nutrients imbalance and deterioration of soil physio-chemical properties causing "soil sickness", which is an important factor affecting crop production (Zhou et al. 2018, Jin et al. 2019, Yu et al. 2019). Several studies have suggested that plant autotoxins cause soil sickness by negatively affecting diversity and abundance of rhizosphere soil microbial community (Zhang et al. 2016, Bai et al. 2019). Phenolics are a kind of allelochemical compounds secreted through root exudates and also found in decomposed plant residues in agricultural soil (Singh et al. 1999). In the rhizosphere, they act as signalling molecules during the initiation of plant-microbe interaction (Mandal et al. 2010, Badri et al. 2013, Khashi u Rahman et al. 2019). Continuous monocropping results in accumulation of these allelochemicals in the soil causing toxicity (Yu et al. 2000, Zhou et al. 2019), which could seriously affect plant growth and development by altering soil microbial communities (Li et al. 2010, Friedman 2017, Jia et al. 2019). For example, Zhou et al. (2017) found that continuously monocropped cucumber increased the abundance of plant pathogenic fungi and decreased that of...
beneficial bacteria when compared with cucumber (Cucumis sativus L.) grown in a crop rotation system. Continuous monocropping of cucumber, a popular greenhouse vegetable crop, is common in most parts of the world. Several studies have found a higher concentration of phenolic acids in monocropped cucumber rhizosphere correlated with more soil-borne diseases and reduced plant growth (Yu et al. 2003, Ye et al. 2006, Zhou et al. 2018).

Studies on the effects of exogenously applied phenolic acids on rhizosphere soil microbial communities showed conflicted results. For example, an exogenously applied phenolic compound, vanillic acid, decreased the abundance of Bacillus and Pseudomonas spp. (Zhou and Wu 2018), while increased that of Fusarium spp. in cucumber rhizosphere (Chen et al. 2018). Manually applied syringic acid decreased as well as increased community abundance of some bacterial species, and the same trend was noticed for fungal communities (Wang et al. 2018). Hence, the response of different microbial communities to certain phenolic acids needs more investigations to improve our knowledge of rhizosphere functioning. Trichoderma spp. are filamentous, opportunistic, avirulent plant symbionts, and parasites of many soil-borne fungi (Harman et al. 2004). They are a prolific producer of numerous biologically active compounds, including secondary metabolites and cell wall degrading enzymes, and have the ability to protect the plant from pathogens in different soil conditions (Vinale et al. 2008, Contreras-Cornejo et al. 2016). However, the response of Trichoderma spp. against phenolic acids during soil sickness is not well documented. The great variability in responses of microbial communities against phenolic acids suggests the requirement of more studies to investigate the response of specific microbial community against specific phenolic acid.

Here we used polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and real-time PCR techniques to show how exogenously applied p-coumaric acid, and ferulic acid affects community structure and abundance of Trichoderma spp. The current study was aimed to investigate the effects of exogenously applied ferulic acid and p-coumaric acid on Trichoderma spp., and how it responds to these phenolic compounds.

**MATERIAL AND METHODS**

**Greenhouse experiment.** The seeds of cucumber (cv. Jinlv 3) were germinated in the soil (moisture of 50 ± 5% of water holding capacity) collected from an open field, which was covered with grasses and undisturbed for ≥ 15 years, in the Horticulture Experimental Station of Northeast Agricultural University, Harbin, China (45°41’N, 126°37’E). The texture of soil was sandy loam, contained 3.67% soil organic carbon (SOC), 89.02 mg/kg available nitrogen, 63.36 mg/kg Olsen phosphorous, and 119.15 mg/kg available potassium, with the electrical conductivity (1:2.5 w/v) 0.33 mS/cm and pH H₂O 7.78. Seedlings were transplanted with two cotyledons to the pots containing 150 g of soil (one plant per pot). Seedlings were grown in a greenhouse (temperature: 32 °C day/22 °C night; 60–80% relative humidity; 16 h light/8 h dark), and no fertiliser was applied.

Cucumber seedlings were treated from the one-leaf stage with different concentrations (0, 0.02, 0.05, 0.1, and 0.2 µmol/g soil dry weight (DW)) of both ferulic acid and p-coumaric acid five times a day after every two days. In total, each treatment W, T1, T2, T3, and T4 received a final concentration of 0, 0.1, 0.25, 0.5, and 1 µmol/g soil DW, respectively. Each treatment was done in triplicate with five plants in each replicate. Since the soil pH is a dominant factor for the regulation of soil microbial communities, so it was adjusted to 7.0 with 0.1 mol/L NaOH (sodium hydroxide) solution (Fierer and Jackson 2006). Seedlings treated with distilled water were considered as control (W). To maintain a constant weight of pots, soil water contents were adjusted with distilled water every two days.

**Rhizosphere soil sampling and DNA extraction.** After ten days of the first application of phenolic acids, the samples of cucumber rhizosphere soil were collected from five plants of each replicate, as described by Zhou et al. (2019). The cucumber seedlings were carefully taken out from the pots, and the soil loosely attached with roots was removed by gently shaking the seedlings. Then, the soil tightly cohering with roots was carefully removed using a sterile brush, considered as rhizosphere soil. After sieving (2 mm), these soil samples were immediately stored at −70 °C until the DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) following manufacturer’s instructions.

**PCR-DGGE analysis.** The PCR-DGGE method was used to analyse the community structure of Trichoderma spp. in cucumber rhizosphere soil. The Trichoderma spp. partial ITS regions were amplified using nested PCR protocol. Primer sets used for Trichoderma partial ITS regions were ITS1F (CTTTGGTCATTTAGAGGAAGTAA/ITS4 GCTGTCGGTGAACAAGG) and ITS2-F-GC (ACTCCAAACCCAATGTGAA) and ITS2-F-GC.
(CGCCCGGGGGCGCGCCCCGGGCGGGGCACGGGGGGACTCCCAAACCCAATGTGAA)/ITS
Tr (TGTGCAAACTACTGCGCA) (Meincke et al. 2010) in the first and second round of amplification, respectively. DGGE analysis of Trichoderma spp. was performed on 6–9% (w/v) acrylamide gel with a 30–60% denaturant gradient (Zhou et al. 2019). Gels were run in 1 × TAE (tris acetate EDTA) buffer for 12 min at 60 °C and 80 V with a DCode universal mutation system (Bio-Rad Lab, USA). Soon after electrophoresis, the gels were stained with 1 : 300 (v/v) GelRed (Bio-tium, USA) nucleic acid staining solution for 20 min. An AlphaImager HP imaging system (Alpha Innotech Corp., USA) was used to photograph DGGE profiles under UV light.

Quantitative PCR assay. To analyse the abundance of Trichoderma spp., the SYBR Green qPCR assay was used in the IQ5 real-time PCR system (Bio-Rad Lab, USA). The Trichoderma spp. partial ITS regions were quantified with a primer set of uTf (AACGTTACCAAACTGTTG)/uTr (AAGTTCAGCGGGTATTCCT) (Hagn et al. 2007) as described before by Drigo et al. (2009). The standard curves were generated with a ten-fold dilution series of plasmids containing soil samples partial ITS regions. To determine the initial copy number of target genes, the threshold cycle (Ct) values of each sample were compared with the standard curve. Sterilised water was used as a negative control to replace the template, and all the treatments were compared with control. The amplification of all samples was performed in triplicate, and the product specificity was confirmed using melting curve analysis and agarose gel electrophoresis.

Statistical analysis. The band patterns of DGGE profiles were analysed with Quantity One V4.5 (Bio-Rad Lan, USA), while the band intensity and position were determined automatically. In order to minimise the influence of loaded DNA concentrations among samples, the density value of every band was divided by the average band density of the lane (Zhou et al. 2019). To compare the banding patterns between samples with normalised data, principal component analysis (PCA) was done using Canoco for Windows 4.5 software (Plant Research International, Wageningen, the Netherlands), as described earlier by Zhou et al. (2019). The microbial diversity index, i.e., the number of bands (S), Shannon-Wiener index (H) and evenness index (E) were calculated as previously described by Zhou et al. (2019). The data were analysed by analysis of variance, and means were compared based on the Tukey HSD (honestly significant difference) test at P ≤ 0.05.

RESULTS AND DISCUSSION

Effect of ferulic acid and p-coumaric acid on Trichoderma spp. abundance. The results of quantitative PCR analysis showed that all the treatments of ferulic acid significantly increased the abundance of Trichoderma spp. in cucumber rhizosphere (P < 0.05). The abundance of Trichoderma spp. increased with the increase in the concentration of ferulic acid (Figure 1). Cucumber seedlings treated with 1.0 µmol/mg soil DW of ferulic acids had the highest abundance of Trichoderma spp. followed by 0.5, 0.25, and 0.1 µmol/g soil DW, respectively. Meanwhile, p-coumaric acid didn’t have any effect on the abundance of Trichoderma spp. in the cucumber rhizosphere, as there was no difference between p-coumaric acid and distilled water treated seedlings (P < 0.05).

Firstly, it was considered that soil sickness caused by continuous monocropping is due to the accumulation of autotoxins in the soil, which affects plant growth.
and development by nutrient imbalance (Olk et al. 1996). Later on, subsequent studies revealed that this accumulation of phenolic acids can also affect plants by altering their soil microbial communities (Eisenhauer et al. 2017, Zhou et al. 2018). However, a diverse range of results found when responses of microbial communities against certain phenolic acids were tested (Qu and Wang 2008, Wu et al. 2016, Zhou et al. 2017). In agreement with previous findings, we found two phenolic acids affecting the abundance of *Trichoderma* spp. complete differently. Continuous monocropping lowers the soil pH resulting in the accumulation of phenolics in the soil (Bai et al. 2019). These phenolics and their derivatives are toxic to most of the microbial species, and their toxicity is negatively correlated with phenol degradation ability of microbes (Krastanov et al. 2013). Microbes degrade phenolic compounds through an ortho-mechanism (3-oxoadipate pathway), which evolves several enzymes to get carbon and energy for their biochemical processes (van Schie and Young 2000, Agarry et al. 2008).

Harman et al. (2004) suggested that, as an indigenous competent soil fungus, most of the *Trichoderma* spp. can utilise phenolic compounds as a carbon source for their proliferation. These findings explain the phenomenon of the increase in abundance of *Trichoderma* spp. in seedlings treated with ferulic acid in our study. On the contrary, the PCR-DGGE and real-time PCR analysis revealed that *p*-coumaric acid had no effect on community structure and abundance of *Trichoderma* spp. in cucumber rhizosphere. As described earlier, phenolic acids selectively affect types of certain microbial communities in the rhizosphere (Qu and Wang 2008). This is because certain microbial species might use certain phenolic acids as their energy source for proliferation and reproduction, while others not. For example, Chen et al. (2011) found that cinnamic acid (derivate *p*-coumaric acid) was not a prior carbon source of *Trichoderma harzianum* SQR-T037.

**Effect of ferulic acid and *p*-coumaric acid on *Trichoderma* spp. community structure.** The DGGE analysis showed an obvious visual difference between the banding pattern of *Trichoderma* spp. between control and other treatments, and the banding pattern of triplicate of each treatment was consistent (Figure 2A). All seedlings treated with ferulic acid, no matter what concentration, had a greater number of bands compared to the seedlings treated with distilled water; also, there was difference in intensity of co-migrating bands and presence and absence of individual bands among ferulic acid treatments and the control. Cucumber seedlings treated with 1 and 0.25 µmol/g soil DW of ferulic acid had the highest number of bands, while the

![Figure 2](A) The denaturing gradient gel electrophoresis (DGGE) map and (B) principal component analysis (PCA) diagram of *Trichoderma* community treated with ferulic acid. The DGGE profiles of *Trichoderma* community treated with different treatments where PCA analysis can distinguish the control from the ferulic acid treated samples. W – 0, T1 – 0.02, T2 – 0.05, T3 – 0.1 and T4 – 0.2 µmol/g soil dry weight of both ferulic acid and *p*-coumaric acid.
control had the lowest (Table 1). The same trend was observed in the Shannon-Wiener and evenness index of *Trichoderma* spp. among ferulic acid treatments, and when compared with control. PCA plot for the DGGE banding pattern of *Trichoderma* spp. explained 60.6% and 25.9% of variations in the first two PCA axis, respectively. The separation between treatments can be seen in the PCA plot (Figure 2B), suggesting the difference in *Trichoderma* community structure significantly affected by ferulic acid treatments ($P < 0.05$). Moreover, the difference between control, low concentration treatments of ferulic acid (T1, T2), and highest concentrations treatments of ferulic acid (T4) were more obvious (Figure 2B). According to the results of DGGE analysis, *p*-coumaric acid did not affect community structure, and the values of the number of DGGE bands (Figure 3A), Shannon-Wiener and evenness index were statistically non-significant ($P < 0.05$) (Table 1). Furthermore, the area of most of the treatments, except T3, in the PCA plot was in cluster form (Figure 3B).

Cucumber seedlings treated with different concentrations of ferulic acid changed the community structure of *Trichoderma* spp. while *p*-coumaric acid did not change any parameter at all when compared with water treated seedlings. Likewise, some previous studies demonstrating changes in community structure of fungal species by phenolic acid treatments (Blum et al. 1999,

![Figure 3](https://example.com/figure3.png)

**Figure 3.** The denaturing gradient gel electrophoresis (DGGE) map of *Trichoderma* community treated with (A) *p*-coumaric acid and (B) principal component analysis (PCA) diagram. The DGGE patterns of *Trichoderma* community with different treatments were almost similar. W – 0, T1 – 0.02, T2 – 0.05, T3 – 0.1 and T4 – 0.2 µmol/g soil dry weight of both ferulic acid and *p*-coumaric acid.
Qu and Wang 2008, Zhou et al. 2017, Chen et al. 2018), our results showed that ferulic acid treatments significantly increased the number of DGGE bands, Shannon-Wiener and evenness index of Trichoderma spp. in cucumber rhizosphere. It is now well understood that the accumulation of phenolic acids in soil due to long term cultivation of the same crop is the main factor of soil sickness (Wu et al. 2016, Zhou et al. 2018, Bai et al. 2019). Our results of Trichoderma spp., which is mostly studied as beneficial plant fungus, response to p-coumaric acid, suggests that the soil sickness caused by p-coumaric acid has no relationship with Trichoderma spp. Moreover, Trichoderma is a group of diverse fungal species containing both plant beneficial (Harman et al. 2004) as well as pathogenic species (Park et al. 2006, Komori-Zelazowska et al. 2007). In our study, ferulic acid might have promoted the abundance of Trichoderma spp. by selectively affecting the strains that are not beneficial to plants. Even so, more studies are needed to confirm the ferulic acid affecting specific species of Trichoderma, and finally affecting the plant health.

Based on the above results, we speculate that the response of Trichoderma spp. against ferulic acid and p-coumaric acid was phenolic acids specificity-based. The different effects of two phenolic acids on Trichoderma spp. might be due to their specific chemical properties, i.e., different functional groups or number of carbon atoms. For example, as compared to p-coumaric acid, ferulic acid has an extra functional group (-OCH₃) and a greater number of carbon atoms. Since soil microorganisms largely interact with phenolic compounds for their carbon need (Blum 1998), and microbial degradation of phenolic compounds is linked with their chemical structures (Sánchez-Maldonado et al. 2011, Shalaby et al. 2012, Jin et al. 2020b); thus, differences in chemical properties could result in different effects of phenolic compounds on the certain microbial community. These findings suggest that one cannot merely describe phenolic acids effect certain microbial genre positively or negatively. This study has further extended our understandings of soil sickness phenomenon specifying phenolic compounds might have different effects on different microbial communities. Further studies are suggested at the individual species level to explore more of phenolic compounds functioning in soil sickness.

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