1   **Metabolomics and microbiome reveal impacts of rhizosphere metabolites on alfalfa**

2   **continuous cropping**

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Abstract

Alfalfa long-term continuous cropping (CC) can pose a serious threat to alfalfa production. However, the mechanism of alfalfa CC obstacle is unclear as of today. In this study, we determined physic-chemical property, microbial population structure, and metabolite differences of alfalfa rhizosphere soils with CC for 1, 7, and 14-years based on analysis of metabolomics and microbiomics. Shifts of functional microorganisms in rhizosphere soil were analyzed, key metabolites and their effects on alfalfa seeds, seedlings and root rot pathogens were assessed. Based on anlysis, p-coumaric acid and ferulic acid on alfalfa seed and seedling growth and root rot pathogens were basically consistent with the influence of CC obstacles in the field. With the increase of CC years, the microbial community in soils changed from fungal to bacterial, and beneficial microorganisms decreased with the increase of CC years, which echoed the performance of alfalfa CC obstacles. The autotoxicity of p-coumaric acid was the strongest.This study fully proved that the continuous accumulation of autotoxic substances in alfalfa rhizosphere was the key factor causing alfalfa CC obstacles.

Keywords: Alfalfa continuous cropping obstacle/ Root rot/ Metabolomics/ Microbiomics/ Autotoxic substances

Introduction

Alfalfa (*Medicago sativa* L.), also called lucerne or purple medic, is a perennial, clover-like, leguminous plant of the pea family (Fabaceae). It is widely grown primarily for hay, pasturage and silage in the United States, Europe and Asia (Graham & Vance, 2003) and is the main animal feed. The Ministry of Agriculture of the People's Republic of China reported that cultivated area of alfalfa in China was 4.7 million ha with a production of 32.17 million tons from the end of 2015 to 2017. Among them, the commercial alfalfa planting area was 0.43 million ha, and the high-quality alfalfa planting area was 0.21 million ha with a production of 1.8 million tons. It was anticipated that the planting area of high-quality alfalfa in China will increase by 0.2 million ha by 2020 with a production of 3.6 million tons. However, with the continuous expansion of alfalfa planting area, alfalfa production capacity decreased year by year in fields continuously planted for more than 4

39   years. Reduced production capacity was reflected in that root activity declined gradually, seed germination rate

40   decreased seriously, root rot incidence increased year by year, and it was difficult to survive resowing or

41   replanting (Rong et al, 2016) This phenomenon is a typical continuous cropping (CC) obstacle of alfalfa.

42   There are two main explanations on the formation of CC obstacles of alfalfa in the literature. The first

43   viewpoint is that alfalfa is a crop with deep root system and high water and fertilizer consumption. After

44   continuous growth for many years, it will lead to soil water and fertilizer deficit, which causes a large area

45   decline of alfalfa growth (Wu et al, 2015; Molero et al, 2019). Li et al. (2010) and Xia et al. (2015) also

46   believed that CC of alfalfa led to deterioration of soil physical and chemical properties that are difficult to

47   restore. However, other scholars hold different points of view. Li et al. (2010) found that planting alfalfa in the

48   same land for more than 10 years could lead to serious land degradation and significant decline of alfalfa yield

49   in the Loess Plateau. Viliana & Ognyan (2015) stated that certain numbers of roots would disappear after each

50   harvest in the CC process of alfalfa, which could provide organic materials for the soil and increase soil

51   organic matter contents. Jiang et al. (2007) reported that soil quality deteriorated but alfalfa yield increased in

52   the first nine years, while soil quality tended to recover but alfalfa yield decreased in the following years. Our

53   field studies also indicated that CC obstacle still occurred even if fertilizers and water were sufficient (Li, Y.

54   G., unpublished). These studies demonstrated that soil moisture and fertility were not the primary factors

55   causing the problems in alfalfa production, such as slow seed germination, delayed returning green, poor

56   growth, yield and quality decline, and severe root rot in CC for more than 4 years.

57   Another explanation is that the continuous accumulation of autotoxic allelochemicals causes CC obstacle of

58   alfalfa. Allelopathy is defined as the direct/indirect harmful/beneficial effect via the production of chemical

59   compounds that escape into the environment (Rice, 1984). Allelopathy is a biological phenomenon by which

60   an organism produces one or more biochemical substances that influence the germination, growth, survival,

61   and reproduction of other organisms in the same community (Zuo et al, 2015). It was reported that alfalfa

62   produced a number of useful phytochemicals, including soyasapogenol glycoside B (Wyman-Simpson et al,

63   1991), medicarpin and isoflavonoid (Miller, 1988), chlorogenic acid, ferulic acid, p-hydroxybenzoic acid,

64   caffeic acid, coumarin, ferulic acid (Rong et al, 2016; Abdul-Rahman & Habib, 1989), amic acid,

65   hydroxybenzoic acid, coumarin and tricinon (Zheng et al, 2018). These phenolic acids had inhibitory effects on

66   plant seedlings, plant photosynthesis and respiration (John & Sarada, 2012; Li et al, 2012; Zhang et al, 2016;

67   Biumal et al, 2019). Chung et al. (2000) reported that chlorogenic acid was involved in alfalfa autotoxicity.

68   Rong et al. (2016) found that contents of coumarin, ferulic acid, chlorogenic acid and caffeic acid varied in 18

69   alfalfa varieties, with coumarin and chlorogenic acid being significantly higher than ferulic acid and caffeic

70   acid. In other studies, phenolic acid such as p-coumaric acid inhibited photosynthesis and enzymatic activities

71   of PG1, CG6PDH, AID and OPPP, which was detrimental to plant and root growth and altered morphological

72   and physical structures of alfalfa roots (Zheng et al, 2018; Rong, 2017). These studies suggested that

73   continuous accumulation of autotoxic substances in alfalfa rhizosphere might be the primary factor causing

74   alfalfa CC obstacle; however, direct evidence supporting the theory is lacking.

75   Methods available for studying CC obstacle, include multi-omics, such as high throughput isolation

76   (culturomics), analyzing structural and functional changes of plant rhizosphere microorganisms

77   (microbiomics), targeting the taxonomic composition (metabarcoding), addressing the metabolic potential

78   (metabarcoding of functional genes, metagenomics), and analyzing components of plant rhizosphere exudates

79   (metabolomics). Through metabolomics, we can quickly understand the metabolic changes of organisms under

80   the stimulation of different biological factors and environmental factors, search for biomarkers with the

81   purpose of identifying metabolites related to various diseases and environmental exposure (Nicholson &

82   Lindon, 2008, Liu et al, 2018). Through microbiomics, we can have a comprehensive understanding that plant

83   surfaces and interior parts are populated by myriads of bacteria, fungi and microbes from other kingdoms

84   which can have considerable effects on plant growth, disease resistance, abiotic stress tolerance and nutrient

85   uptake (Xie et al, 2019). In CC systems, the same crop root secretions may not only affect plant growth, but

86   also lead to simpler microbial community structure, which may negatively affect agroecosystems especially in

87   terms of the aggravation of pathogens and soil-borne fungi. Plant-microbial interactions can have positive or

88   negative effects on plant growth through a variety of mechanisms. Root exudates are one of the most important

89   chemical signals in determining whether the interactions are benign or harmful (Bais et al, 2006). Identification

90   of major differences in root exudates may be helpful in understanding changes of rhizosphere microbial

91   community structure (Brown et al, 2020). The combination of metabolomics and microbiomics can be a

92   powerful tool to analyze the mechanism of CC obstacles. Lin et al. (Lin et al, 2015) reported that the

93   accumulation of allelochemicals in rhizosphere soil increased harmful microorganisms and decreased

94   beneficial microorganisms, resulting in imbalance of microbial community structure and degradation of soil

95   ecological function. Zhao et al. (2018) indicated that continuous coffee cultivation reduced potentially

96   beneficial microbes in soil. Li et al. (2014b) showed that peanut root exudates promoted the growth of root rot

97   pathogens *Fusraium oxysporum* and *Phoma* sp. and inhibited the growth of beneficial bacteria, such as

98   *Mortierella elonate* and *Trichoderma* sp. However, the advanced metabolomics and microbiomics technologies

99   have not been used in research of CC obstacles of alfalfa to elucidate mechanisms involved in the complex

100 disease system.

101   The objectives of this research were: 1) to determine the role of continuously accumulation of autotoxic

102 substances in CC obstacle of alfalfa; 2) to identify key metabolites causing CC obstacle and determine their

103 effects on alfalfa seed germination and seedling growth; and 3) to evaluate the effects of key metabolites on

104 root rot pathogens and diseases and rhizosphere microecology.

105

106 **Results**

107 **Soil nutrients and enzyme activities**

108 Most of the nutrient contents tested in the 7 and 14-year rhizosphere soils were lower than the 1-year

109 rhizosphere soil, including N, P, K, B, Fe, Mn, and Mg, but Cu and Zn did not decrease with the increase of

110 CC years (Table 1). There were significant differences in soil enzyme activities in the rhizosphere soils of

111 different cropping years ($P < 0.05$, Fig. 1). Polyphenol oxidase, neutral phosphatase and sucrase activities

112 decreased with the increase of CC years, while urease increased in 7-year and decreased in 14-year rhizosphere

113 soils.

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115 **Soil extracts from alfalfa rhizosphere soil on alfalfa growth**

116 When alfalfa seeds were treated with alfalfa rhizosphere soil extracts, the germination rate in treatment with

117 rhizosphere soil extract of alfalfa planted for 1 year was equivalent to the nontreated control, which was

118 significantly higher than those planted for 7 and 14 years ($P < 0.05$, Fig. 2 *A* and *C*). Height of alfalfa seedlings

119 in the 1-year treatment, but not the 7 and 14-year treatments, was significantly greater than the control (Fig. 2

120   *B* and *D*). However, root length of the 1, 7 and 14-year treatments was not significantly different from the

121   control.

122

Metabolomics analysis of alfalfa rhizosphere soils

124   The peak with detection rate below 50% of RSD >30% was removed from the QC samples (Dunn et al, 2011).

125   Principle Component Analysis (PCA) and least aquares-discriminant analysis (PLS-DA) were performed. A

126   total of 161 different metabolic compounds were identified from rhizosphere soils of the 1, 7, and 14-year

127   treatments (Fig. 3), which were classified according to their chemical property as sugar compounds (26), sugar

128   acids (3), sugar alcohols (6), short-chain organic acids (16), long-chain organic acids (31), nuleotides (12),

129   amino acids (14), esters (6), alcohols (16), and others (31). There were significant differences in the peak

130   values of 58 metabolites from the rhizosphere soils. Combing VIP > 1 and independent sample T test ($P <$

131   0.05), there were 52 differential metabolites down-regulated and 6 differential metabolites up-regulated (Table

132   S1). Among them, vanillic acid, p-hydroxybenzoic acid, ferulic acid, and p-courmaric acid increased

133   significantly with the increase of CC years, and accumulation of p-hydroxybenzoic acid and p-coumaric acid

134   was more significant with the increase of alfalfa CC years (Table 2).

135   The effects of four phenolic acids on alfalfa plant were evaluated at concentrations of 10, 25, 50 and 100

136   μg/mL. Inhibitory effect on seed germination and plant growth was as follows: p-coumaric acid > ferulic

137   acid > vanillic acid and p-hydroxybenzoic acid, with higher concentrations having greater inhibition than a

138   lower concentration (Fig. 4). P-coumaric acid reduced root length significantly at all concentrations, compared

139   to the control, and the other three compounds reduced root length significantly at higher concentrations (Fig. 4

140   *E-H*). Higher concentrations of ferulic acid and p-hydroxybenzoic acid reduced plant height significantly

141   compared to the control, but vanillic acid did not reduce plant height at all concentrations (Fig. 4*E*).

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Key metabolites from alfalfa rhizosphere soils on alfalfa root rot pathogens

144   Three fungal pathogens *Fusarium trinctum*, *F. acuminatum* and *F. oxysporum* that cause alfalfa root rot were

145   used to assess the relationship between metabolites of alfalfa rhizosphere and root rot of alfalfa. Extracts of

146   rhizosphere soil in 14-year alfalfa CC significantly enhanced mycelial growth of *F. trinctum*, *F. acuminatum*

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147 and *F. oxysporum* when used at 1%, 5% and 10% compared to 1-year and the water control (Fig. 5). Extracts

148 of rhizosphere soil in 7-year alfalfa CC also enhanced mycelial growth of the pathogens significantly when

149 used at higher concentrations. The effects of four phenolic acids on mycelial growth of the fungal pathogens

150 were evaluated. They enhanced mycelial growth with effects in the following order: p-coumaric acid > ferulic

151 acid > vanillic acid >p-hydroxybenzoic acid (Fig. 6). Higher concentrations of the phenolic acids had greater

152 effects in enhancing mycelial growth of the fungal pathogens. When tested at 10, 25, 50 and 100 µg/mL, the

153 four phenolic acids at higher concentrations also significantly enhanced conidial germination and production of

154 *F. acuminatum* and *F. oxysporum* (Fig. 7). Among the phenolic acids, p-coumaric acid had the greatest

155 promoting effect on conidial germination and production. When inoculated with *F. trinctum*, *F. acuminatum*

156 and *F. oxysporum* in greenhouse studies, severity of alfalfa root rot treated by p-coumaric acid increased

157 significantly with 50 μg/mL having greater effect than 10 μg/mL (Fig. 8). Growth and development of

158 seedlings were also suppressed by p-coumaric acid.

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Alfalfa CC on alfalfa rhizosphere microecology

161 Microbial community sequences of the rhizosphere soil samples were analyzed on Illumina Miseq PE300

162 platform and divided by OTUs. Principle Component Analysis (PCoA) based on detected OTUs showed that

163 there were significant differences in community composition of the rhizosphere soils, and Anova analysis

164 showed that there were significant differences among groups ($P < 0.05$, Fig. 9 *D1* and *E1*). The diversity of

165 bacterial community was high and the number of OTUs decreased with the CC increase (Fig. 9 *D2*). The

166 diversity of bacterial community of 14-year CC was the lowest, while the diversity of fungal community of

167 1-year cropping was the lowest (Fig. 9 *E2*). Small changes in root exudates can lead to changes in soil

168 microbial structure (Ling et al, 2011). This change could lead to soil microecological damage as well as disease

169 and pest problems (Liu et al, 2020). These results seem to indicate that CC can change the microbial

170 composition of soil from bacterial-dominated to fungal-dominated (Lin et al, 2015).

171 Most OTUs of bacteria were *Actinobacteria* (35.55%, 37.54%, and 40.28% for 1-year, 7-year and 14 year

172 CC respectively), *Acidobacteria* (24.07%, 18.49%, and 19.53% for 1-year, 7-year and 14-year) and

173 *Proteobacteria*, and a small proportion belonged to *Gemmatimonadetes*, *Verrucinobacter*, *Nitrospiraceae*, and

174   *Bacteroidetes* (Fig. 10*D*). *Bacillus* is considered an important soil conditioner and can be used as a biological

175   control agent (Li et al,, 2018). In our present study, the abundance of *Bacillus* decreased in years and then

176   increased in 14 years. Most OTUs of fungi belonged to *Ascomycota* (56.14%, 65.57%, and 73.72% for 1, 7,

177   and 14-year CC, respectively), *Basidiomycota*, *Mortierellomycota*, *Glomeromycota*, and *Chytridiomycota* (Fig.

178   10*E*).

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180   **P-coumaric acid on rhizosphere microbial communities**

181   In order to verify the correlation between microbial population changes and metabolites secreted in the

182   rhizosphere, alfalfa rhizosphere soil was treated with p-coumaric acid, the most influential metabolite secreted

183   by alfalfa. The results showed that significant changes occurred in the bacterial and fungal communities

184   (Fig.11 *D1* and *E1*). The number of OTU in bacterial and fungal communities decreased when treated with 10

185   μg/mL p-coumaric acid but increased when treated with 50 μg/mL p-coumaric acid. The number of OTU in

186   soil bacterial community treated with p-coumaric acid was less than that of the control (Fig.11 *D2* and *E2*).

187   The biodiversity of bacterial community was the highest when treated with 10 μg/mL of p-coumaric acid, the

188   lowest with 50 μg/mL, and the diversity of soil fungal community increased gradually with the increase of

189   p-coumaric acid concentration, which was consistent with the previous results of continuous cropping.

190   However, the number of microorganisms in alfalfa rhizosphere soil in the pots treated with p-coumaric acid

191   was lower than that in the field.

192   Analysis of microbial species and functions indicated that the *Gemmatimonadetes* decreased with increasing

193   concentration of p-coumaric acid (Fig.12*D*). The relative abundance of bacterial species decreased to different

194   degrees, with 8 main bacterial species in the top 20 species showing a decreasing trend, 6 bacterial groups

195   showing the trend of decreasing when treated with 10 μg/mL p-coumaric acid and increasing with 50 μg/mL,

196   and 5 groups of bacteria showing the trend of increasing with 10 μg/mL and decreasing with 50 μg/mL (Fig.

197   12*F*). *Sphingomonas* increased with the addition of p-coumaric acid, which was consistent with the

198   experimental results described above. *Sphingomonas* and *Gemmatimonas* were related to nitrogen metabolism

199   and transformation, as well as the change of NH_4^+-N and N_2O contents (Nathanae et al, 2009; Shen et al,2014,

200   Li et al, 2017). *Candidatus Solibacter* is able to decompose organic matter, and the results showed it decreased

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201   with the increase of p-coumaric acid concentration (Zak et al, 1996). *Bryobacter* promoted soil carbon cycle

202   (Li et al, 2012), which showed a trend of increasing at first and then decreasing, and was lower in the

203   p-coumaric acid treatment (50 μg/mL) than the control soil (Fig.12*E*).

Discussion

205   The soil enzyme is a key criterion for evaluating different residue covering approaches, as it is an important

206   indicator of soil quality and function. Sucrase can reflect the conversion ability of organic carbon (Cantarella et

207   al, 2018). As the CC years increase, the conversion of organic carbon gradually decreases, which is consistent

208   with studies of Liu et al. (2017). Soil urease reflects the transformation ability of soil organic nitrogen to

209   available nitrogen and the supply ability of soil inorganic nitrogen (Tawaraya et al, 2014). Phosphatase can

210   catalyze the hydrolysis of soil monophosphate to form inorganic phosphorus, which can be absorbed by plants,

211   and soil phosphatase activity can be used to characterize the state of soil phosphate (Hu et al, 2015). Our

212   present study indicated that the activity of soil phosphatase transformation decreased with the increase of CC

213   years. In the meantime, the content of phosphorus in soil decreased with the increase of CC years. Soil catalase

214   activity indicates its ability to remove the toxicity of hydrogen peroxide, which could reflect soil quality and

215   the total metabolic activity of soil microorganisms (Zhang et al, 2012). Overall, soil quality became worse with

216   the increase of CC years of alfalfa.

217   Development of alfalfa seedlings needs the support of external nutrition. In the study plant height and root

218   length from seeds treated with rhizosphere soil extracts decreased with the increase of CC years, which might

219   be because soil nutrient condition gradually became worse. However, theoretically nutrition for seed

220   germination is provided by the nutrition stored by the seed itself and there is little need for extra nutrition. The

221   richness of soil nutrients only affects the growth of seedlings after germination but does not affect seed

222   germination rate (Meng et al, 2006). Our present study provides two evidences that do not support the

223   view that the main factors of alfalfa CC obstacle were the lack of soil nutrients. One was that seed germination

224   does not need external nutrients, and at least the germination rate of alfalfa seeds treated by soil extracts from

225   the 1, 7, and 14-year CC should be similar to that of the water treatment control. However, it is interesting that

226   seed germination rate decreased significantly with the increase of CC years. Another evidence was that

227   nutrients in the soil extracts from the three alfalfa cropping years were richer than the water control. In theory

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228   the soil extracts of the three cropping years should have significant effects on the growth of alfalfa seedlings

229   compared to the control. However, our results showed that the effects of soil extracts from CC for 7 and 14

230   years on seedling growth were similar to that of the control. These results indicated that lack of nutrients in

231   alfalfa rhizosphere soil was not the key factor resulting in alfalfa CC obstacles.

232   As our results, the accumulation of phenolic acids in the soil after CC significantly affects the rhizosphere

233   ecosystem, for instance, by inducing changes in microbial populations, soil enzyme activity and nutrient

234   cycling (Halvorson et al, 2009; Chen et al, 2020). Many perennial and annual crop species are threatened by

235   CC problems associated with reduced plant growth and vigor as well as reduced crop yields and quality (Chen

236   et al, 2012; Li et al, 2018; Li & Liu, 2018). In fact, previous studies have shown that phenolic acids are a major

237   secondary metabolite of CC disorder (Muscolo & Sidari, 2006). In studies on cucumber, strawberry, tobacco

238   and Rehmannia, soils continuously planted were found to contain self-toxic substances, phenolic acid, which

239   repressed growth of the same plant (Li et al, 2012; Wu et al, 2009; Chen et al, 2011; Li et al, 2015b). With the

240   extension of CC years, the concentration of phenolic acid in the soil increased gradually (Huang et al, 2013).

241   Qu and Wang (Qu & Wang, 2008) reported that two phenolic acids from soybean root exudates,

242   2,4-di-tert-butylphenol and vanillic acid, had significant negative effect on microbial communities and soybean

243   monoculture. Li et al. (2012) also found that the extracts from rhizosphere soil of Ginseng CC had significant

244   inhibition allelopathy on the growth of radicle. These are consistent with our findings about the inhibitory

245   effects of the phenolic acids on alfalfa. With the increase of alfalfa CC years, the contents of four phenolic

246   acids (vanillic acid, p-hydroxybenzoic acid, ferulic acid and p-courmaric acid) increased significantly (Table 2).

247   Therefore, we thought that the occurrence of alfalfa CC obstacle may be related to the phenolic acids.

248   Crop roots respond to pathogen infections by changing the amount and composition of root exudates

249   (Lanoue et al, 2010). Studies showed that cucumber CC usually led to the accumulation of soil-borne

250   pathogens such as *Fusarium* spp. (Zhou & Wu, 2012). In alfalfa CC, we also found that root rot caused by

251   diverse pathogens increased with the increase of alfalfa CC years. Wu et al. (2015) reported that a mixture of

252   phenolic acids promoted the growth of *F. oxysporum* hyphae, spore formation and production. Zhou et al.

253   (2012) indicated that the amount of p-coumaric acid from cucumber could increase the number and population

254   density of *F. oxysporum* in soil and disease incidence in the field. A number of studies also demonstrated that

255   CC significantly increased levels of fungal pathogens causing root diseases of *Rehmannia glutinosa*, soybean

256   and cucumber (Wu et al, 2015; Guo et al, 2011; Zhou & Wu, 2012a). These studies are in agreement with our

257   research that alfalfa root rot was getting more severe with the increase of CC years. Our present study further

258   confirmed that metabolites secreted by alfalfa rhizosphere, such as p-coumaric acid, had strong effects on

259   alfalfa root rot and may be the key factor of alfalfa CC obstacle.

260   Soil bacteria are responsible for decomposing organic matter into inorganic matter and thus maintaining soil

261   fertility, so we did a differential analysis of soil bacterial flora for different years of CC (Fig. 10*F*). The results

262   show that *Gaiella*, *Pseudonocarida*, *Mycobacterium* and *Bradyrhizobium* in the soil samples increased

263   gradually with the increase of CC years, and *Solirubrobacter* decreased with the increase of CC years. The

264   relative abundance of *Sphingomonas* was less than 1% in 1-year and more than 1.4% in 14-year treatments,

265   showing an increase with CC. Other studies have shown that *Sphingomonas* was a nutrient-poor

266   nanobacterium, which could adapt to heterotrophic growth under conditions of nutrient depletion (Williams et

267   al, 2009; Kämpfer et al, 2002; Pooja et al, 2010). In this paper, the increase of *Sphingomonas* indicates that

268   after 14 years of continuous cultivation, the rhizosphere soil presented a state of nutrient depletion. This is

269   consistent with the results of Chen et al. (2020). The relative abundance of bacteria RB41 was higher than

270   7.2% in 14 years of CC, which was higher than in 1 and 7-years. The acid bacteria may play an important role

271   in remaining the metabolism of soil under long-term low nutrient stress, and could degrade the polymer of

272   plant residues (Aislabie et al, 2006; Fan et al, 2018). *Bradyrhizobium* is a diazotrophic bacterium in soil, which

273   plays an important role in nodule formation, ammonia production and nitrogen fixation symbiosis in legume

274   roots (Masuda et al, 2016; Shiro et al, 2016; Saeki et al, 2017; Siqueira et al, 2017). The difference of relative

275   abundance of *Bradyrhizobium* in three different CC years was significant, which was higher in 14-year CC

276   than in 1-year. Two fungi *Gibberella* and *Metarhizium* were 11.5% in soil after one year of CC and 1-2% after

277   14 years of CC. In the CC process, the bacterial community in soil decreased significantly.

278   It appeared that the change of alfalfa rhizosphere microbial communities treated with p-coumaric acid was

279   basically consistent with that of alfalfa CC obstacle. There were some differences between results in the potted

280   alfalfa study and the actual field conditions, which was probably due to other secondary autotoxic substances.

281   Overall, alfalfa CC had an impact on soil microbial communities, and the accumulation of autotoxins in

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282   rhizosphere soils increased harmful microorganisms and decreased beneficial microorganisms (such as

283   *Gemmatimonadetes* and *Sphingomonas et al*), resulting in imbalance of microbial community and degradation

284   of soil ecological function.

285   And the results of the four phenolic acid treated seeds and pathogens were similar to those treated by soil

286   extracts. The occurrence of CC obstacle was directly related to the four phenolic acids secreted by alfalfa

287   rhizosphere. Among them, the effects of ferulic acid and p-coumaric acid on alfalfa seed germination and

288   mycelium growth were significant. The effects of p-coumaric acid on alfalfa seedling development were

289   obvious, and the effects of ferulic acid on spore production and germination of pathogenic fungi causing alfalfa

290   root rot were significant. Bi et al. (2010) reported that nine phenolic acids, such as p-hydroxybenzoic acid,

291   coumaric acid, and ferulic acid, were detected in commercially grown Ginseng rhizosphere soils, which could

292   inhibit the growth of radicles and buds. Zhou &Wu (2012a) found p-coumaric acid, an autotoxin of cucumber,

293   increased *F. oxysporum* f. sp. *cucumerinum* population densities in soil and the severity of Fusarium wilt under

294   field conditions. Wu et al. (2015) indicated that phenolic acid mixtures promoted hyphal growth, spore

295   formation and production of *F. oxysporum* that causes wilt disease of *Rehmannia glutinosa*. Tao et al. (2018)

296   reported that both p-hydroxybenzoic acid and ferulic acid could inhibit alfalfa seed germination and seedling

297   development. Therefore, we think that the four phenolic acids secreted in the rhizosphere of alfalfa were the

298   main factors causing severe alfalfa root rot in the alfalfa CC obstacle.

299   In this study, we found that the main factors causing CC obstacle were not the lack of nutrients or water in

300   alfalfa rhizosphere soil. Based on metabolomics and microbiology analysis, the effects of certain key

301   metabolites, including p-coumaric acid, ferulic acid and other phenolic acids, on alfalfa seed and seedling

302   growth and root rot pathogens were basically consistent with the influence of CC obstacles in the field. In

303   addition, with the increase of CC years, the microbial community in soil changed from fungal to bacterial, and

304   beneficial microorganisms decreased with the increase of CC years. The effects of the key metabolites from

305   alfalfa rhizosphere on alfalfa seed germination, seedling growth and root rot were further verified, which

306   resulted in similar alfalfa performance as in CC obstacles. Among these key metabolites, the autotoxicity of

307   p-coumaric acid was the strongest. This study fully proved that the continuous accumulation of autotoxic

308   substances in the rhizosphere was the key factor of alfalfa CC obstacle.

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Materials and Methods

Soil Sampling

The study was conducted on an experimental farm located in Dörbets, Daqing, Heilongjiang, Northeast China

(124.25′ E, 46.30′ E) with a continental monsoon climate. The soil type was sandy loam and average depth of

topsoil was about 30 cm. No location had history of hardpan. Soil samples were collected in October 2019

using the five-spot-sampling method from the rhizosphere of alfalfa plants grown in the fields with a history of

alfalfa CC for 1, 7 and 14 years. The soil samples, thoroughly homogenized through a 20-mesh sieve to

remove root debris, were placed in sterile bags and then transferred to liquid nitrogen and stored in ice boxes.

The samples were transported to the laboratory and stored at -80℃ for

metabonomic and microbiological analysis. In the meantime, a portion of the soil samples was dried for

analysis of soil properties. Each treatment had three replicate samples.

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Assessment of soil properties

Physic-chemical properties of the rhizosphere soils, including available P (AP), available N (N), Fe, Mn, Cu,

Zn and EC values, were analyzed at the Soil and Fertilizer Testing Center of Heilongjiang Academy of

Agricultural Sciences (Harbin, China). Enzyme activities were assessed using a soil enzyme activity test Kit

(Suzhou Grace Biotechnology Co., Ltd., Suzhou, China). Specific measurement and analysis followed the

manufacturer's instructions using Multiskan sky (Thermo Fisher Scientific, Waltham. MA, USA). Assessment

of the enzyme activities was as the following: soil urease (S-UE) was measured at 578 nm; neutral phosphatase

(S-NP, G0306W) catalyzes p-nitrophenyl phosphate (pNPP) to produce a yellow product PNP, which has a

maximum absorption peak at 405 nm, and the enzyme activity was measured by the rate of increase of PNP;

solid polyphenol oxidase (S-PPO, G0311W) catalyzes gallic acid to produce gallium, which has a

characteristic light absorption at 430 nm, reflecting polyphenol oxidase activity; solid sucrase (S-SC, G0302W)

catalyzes the degradation of sucrose into reducing sugar and reacts with 3, 5-dinitrosalicylic acid to form

colored metal amides with characteristic light absorption at 540 nm; soil catalase (S-CAT, G0303W) catalyzes

hydrogen peroxide to produce water and oxygen and the remaining hydrogen peroxide reacts with a

13

336 chromogenic probe to produce a colored substance with a maximum absorption at about 510 nm. Each

337 treatment had three replicates and the experiment was conducted twice.

338

Effects of soil extracts on alfalfa seedling and pathogen growth

340 To assess the effects of the autotoxic substances on alfalfa seed and fungal pathogens causing alfalfa root rot,

341 soil extracts from the rhizosphere soil samples were prepared. Ten grams of soil were mixed with 250 mL

342 distilled water and shaken for 24 h (Yang et al, 2009). The supernatant was filtered, distilled and concentrated

343 to 1 mL/g of soil using a rotary evaporator (Strike 300, Guangzhou Wengdi Instruction Co., Ltd, China). The

344 concentrated supernatants were filtered through a bacterial filter and stored at 4℃.

345 Alfalfa seeds were immersed in 1.5% sodium hypochlorite for 10 min, rinsed 5 times with distilled water, air

346 dried, and soaked in the soil extracts (1 mL/g) for 30 min. Treated seeds were placed on sterile filter paper in a

347 petri dish (50 seeds/dish). Two mL of soil extract was added to each dish and the dishes were incubated in a

348 growth chamber at 25℃ with 12-h photoperiod. Alfalfa seeds treated with equal amount of distilled water were

349 used as the control. Each treatment had three replicates and the experiment was conducted twice. Seed

350 germination rate, root length and plant height of alfalfa were measured 7 days after incubation.

351 To assess the effects of soil extracts on pathogens causing alfalfa root rot, three fungal pathogens were used

352 including *Fusarium trinctum* (MH894213), *F. acuminatum* (MK764994) and *F. oxysporum* (MK764964). The

353 fungi were grown on potato dextrose agar (PDA) plates at 25℃ for 5 days, and a mycelium plug (0.7 cm in

354 diameter) was transferred onto PDA plates amended with the soil extract (final concentration V/V: 1%, 5% and

355 10%). PDA plates amended with equal amount of distilled water were used as controls. The plates were

356 incubated at 26℃ for 5 days and colony diameters were measured. Each treatment had three replicates and the

357 experiment was conducted twice.

358

Metabolomics analysis of alfalfa rhizosphere soil

360 The rhizosphere soil samples were extracted with methanol-water (v/v 3:1), ethyl acetate,

361 L-2-chlorophenylalanine, and air dried. The air-dried soil extracts were dissolved in 20 μL methoxyamine salt

362 and 30 μL BSTFA (containing 1% TMCS). Metabolites of the rhizosphere soils were assessed using 7890A gas

14

363   chromatography-time-of-fight mass spectrometry (GC-TOF-MS) with DB-5MS capillary column (Agilent,

364   USA) at Beijing Allwegene Co., Ltd. (Beijing, China). The Chroma TOF software (V 4.3x, LECO) was used to

365   analyze the mass spectrum data, including peak extraction, baseline correction, deconvolution, peak integration

366   and peak alignment (Kind *et al*., 2009). The LECO-Fiehn RTX5 database, including mass spectrometry, match

367   and retention time index match, was used in the qualitative analysis of the substances.

368

369   **Validation of key metabolites**

370   To determine the role of key metabolites in alfalfa CC, the effects of key metabolites from the rhizosphere soils

371   on seed germination, seedling growth, and growth of root rot pathogens *F. trintum*, *F. acuminatum* and *F.*

372   *oxysporum* were determined. The following experiments were conducted:

373

374   ***Effect of key metabolites on alfalfa seed and seedling.*** Alfalfa seeds disinfected as described above were

375   immersed in different concentrations (10, 25, 50, 100 μg/mL) of key metabolites for 30 min. The seeds were

376   placed in a sterile culture dish, covered with two layers of sterile filter paper, dripped with the corresponding

377   concentration of key metabolites (1 mL/two days), and placed in a humidity chamber (>95% RH, 24°C and

378   16/8 h light/dark). Each treatment had three replicates and the experiment was conducted twice. Seed

379   germination rate, root length and plant height were measured 7 days after incubation.

380

381   ***Effect of key matabolites on pathogenic fungi causing alfalfa root rot.*** A mycelium plug (0.7 cm in diameter)

382   of *F. trinctum*, *F. acuminatum* and *F. oxysporum* grown on PDA for 5 days was transferred onto PDA plates

383   amended with different key metabolites at 10, 25, 50, and 100 μg/mL. The plates were incubated at 26°C in

384   dark and colony diameters were measured 5 days after incubation. Plates amended with equal amount of sterile

385   distilled water (SDW) were used as controls. Each treatment had three replicates and the experiment was

386   conducted twice.

387   To evaluate effects of metabolites on spore germination, *F. trinctum*, *F. acuminatum* and *F. oxysporum* were

388   grown on PDA at 25°C until colony diameters were 5 cm or larger. Conidia on the plates were washed with

389   SDW, and the concentration was adjusted to 10^6 spores/mL by counting using a hemocytometer. Different key

15

390   metabolites were added into the conidial suspensions at concentrations of 10, 25, 50 and 100 μg/mL. Conidial

391   suspensions amended with an equal volume of SDW served as a control. Spore suspensions were incubated at

392   25°C until spore germination rates were greater than 10%, and germinated spores were counted by counting

393   100 spores for each treatment in a replicate. To evaluate effects of metabolites on spore production, the three

394   fungal cultures were grown as above. Different key metabolites at concentrations of 0, 10, 25, 50, or 100

395   μg/mL were added to each plate (20 mL/plate). Mycelia on the surface of the PDA plates were scraped off, and

396   liquid on the plates was poured out after 20 min. The plates were then incubated at 26°C for 72 h, and 20 mL

397   of SDW was added to a petri dish to wash the spores off the mycelium (Li et al, 2010). Spore suspension in a

398   dish was collected in a tube (50 mL) and spore concentration was determined using a hemocytometer. Each

399   treatment had three replicates and the experiment was conducted twice.

400

401   **Microbiological analysis of alfalfa rhizosphere soil**

402   Total DNA was extracted from the rhizosphere soils using PowerSoil DNA Isolation Kit (MoBio Laboratories,

403   CarIsbad, CA, USA). DNA concentration was quantified using a NanoDrop^{TM} 2000 spectrophotometer

404   (Thermo Fisher Scientific, Waltham, MA, USA). Research on 16S rRNA/ITS sequencing and sequencing of

405   the complete metagenomic sequence. The primer sets ITS1 (CTTGGTCATTTAGAGGAAGTAA) / ITS2

406   (TGCGTTCTTCATCGATGC)   and   338F   (ACTCCTACGGGAGGCAGCAG)   /   806R

407   (GGACTACHVGGGTWTCTAAT) were used to amplify target regions of fungal and bacterial genes,

408   respectively.

409   The quality-checked DNA samples were then sequenced on Illumina Miseq PE300 platform. To guarantee

410   the quality of data for downstream analysis, Trimmomatic was used to remove raw reads with tail end quality

411   score < 20. Data pre-processing was conducted to obtain a good sequence. Through the sorting operation, the

412   sequences were divided into different groups according to their similarity, and a group was an OTU. All

413   sequences were divided into OTU according to different similarity level, and OTUs under 97% similarity level

414   could be analyzed statistically (Edgar, 2013). The data were extracted based on the OTU clustering results, and

415   the Alpha (Amato et al, 2013) and Beta (Jiang et al, 2013) analyses were carried out using Qiime (Version

416   1.8, http://qiime.org/), uclust (Version 1.2.22, http://www.drive5.com/uclust/downloads1_2_22q.html) and

417   usearch (Version 10.0.240, http://www.drive5.com/usearch/)

418

Validation of effects of p-coumaric acid in greenhouse

420   Alfalfa seeds were surface disinfested as described above and planted in a seedling tray in a greenhouse at

421    $25\pm2°C$. Seven-day-old seedlings were transplanted in pots (5 plants/per pot). Plants in 5 pots were treated

422   with p-coumaric acid 3 days after transplanting at 10, 25, 50, or 100 μg/mL, respectively (1 mL/per plant).

423   Treatment with p-coumaric acid was applied once every three days, and plants in 5 pots treated with SDW

424   were used as controls. Alfalfa rhizosphere soils were collected as described above after treatment with

425   p-coumaric acid for 5 times (i.e., 15 days after transplanting). Soil samples were stored at -80°C for

426   microbiological analysis. Soil DNA extraction and microbiological analysis were as described above.

427

Acknowledgements

434

Author contributions

436   RT performed all experiments. WY and JX provided help of the experiments. YG designed the study and

437   modify the majority of the manuscript. PS provided comments on the manuscript. All authors read and

438   approved the final manuscript.

439

Conflict of interest

441   The authors declare that they have no conflict of interest.

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17

444 **Reference**

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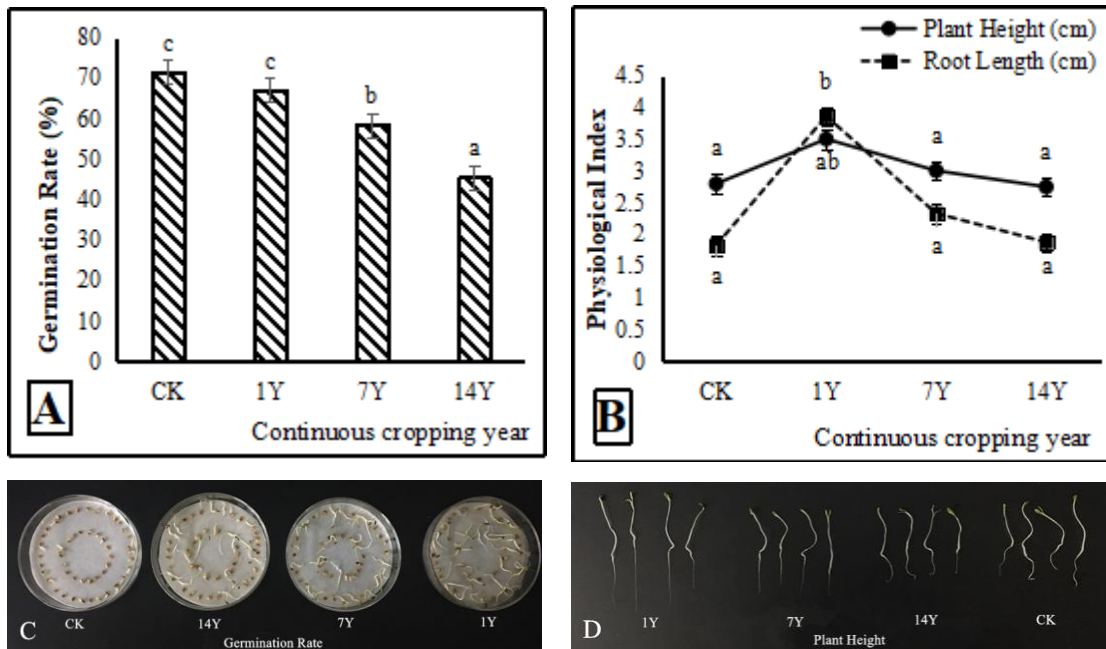
Fig.1. Effects of rhizosphere soil extracts from fields with different years of alfalfa continuous cropping on alfalfa seed germination (A, C) and seedling growth (B, D). Error bars indicate standard errors of the means of three repetitions. Different letters above the bars indicate significant difference according to Duncan's multiple range test ($P = 0.05$).

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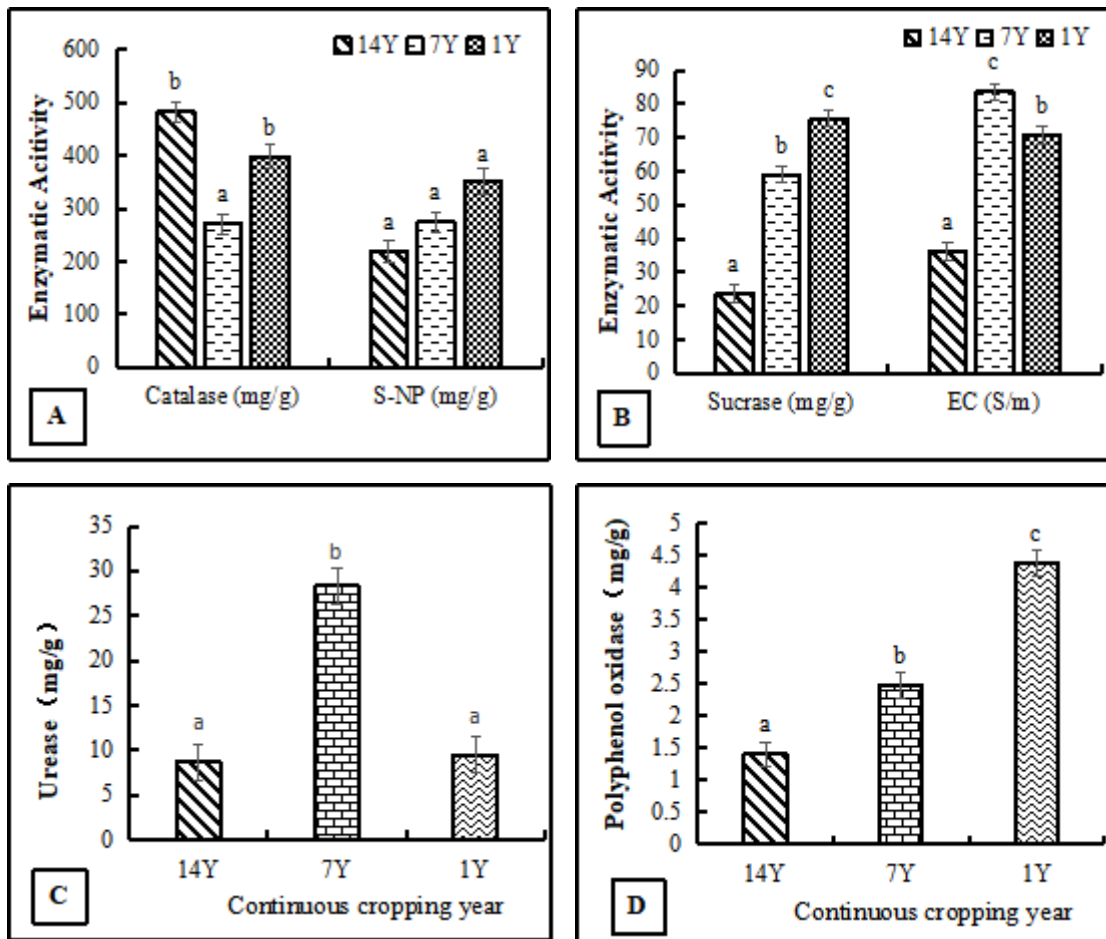
Fig. 2. Changes of enzyme activity, electrical conduction and organic matter content in rhizosphere soils from fields with continuous cropping of alfalfa for 1, 7, and 14 years. A) Catalase and S-NP activity; B) Sucrase activity and electrical conduction; C) Urease activity; D) Polyphenol oxidase activity. Error bars indicate standard errors of the means of three repetitions. Different letters above the bars indicate significant difference according to Duncan's multiple range test ($P = 0.05$).

(A) (B) (C)
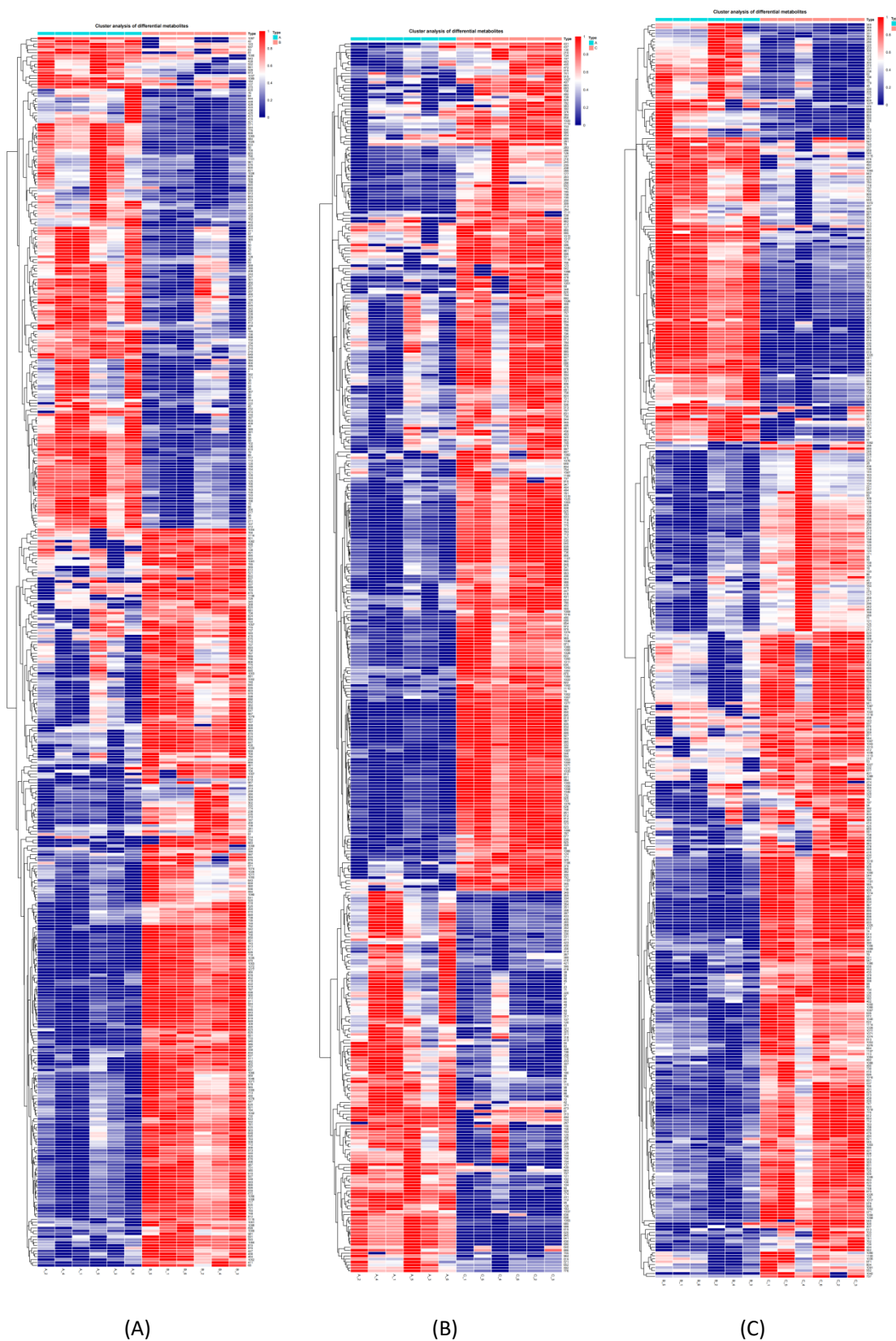
Fig 3. Heatmap analysis of two-year comparison of changes of alfalfa root exudates from fields with

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631   different years of continuous cropping. A) 1-year vs. 7-year; B) 7-year vs. 14-year; C) 1-year vs. 14-year.

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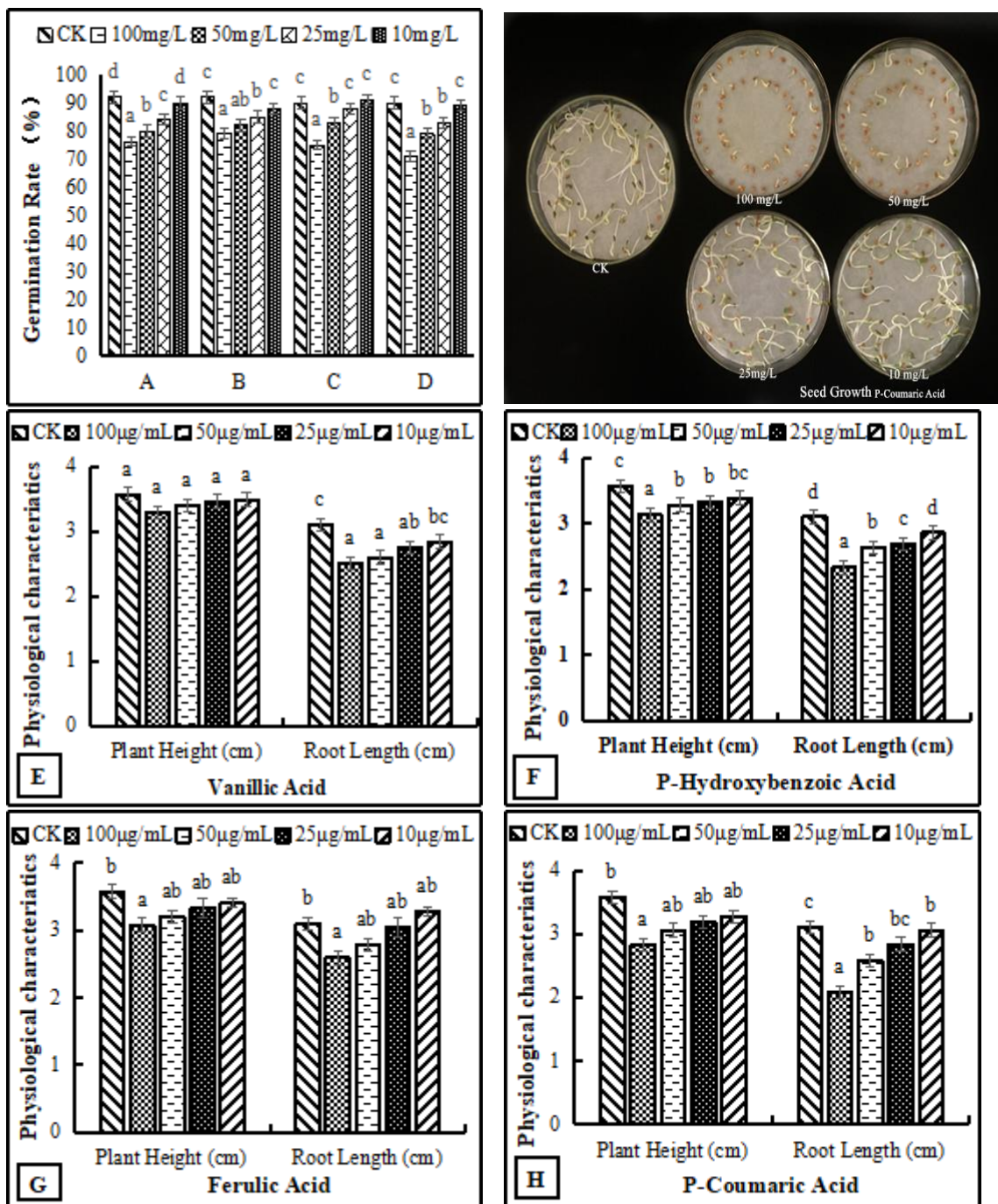
Fig. 4. Effect of different concentrations of phenolic acids on alfalfa germination rate, root length and plant height (E, F, G, H). A) Vanillic Acid; B) P-Hydroxybenzoic Acid; C) Ferulic Acid; D) P-Coumaric Acid. Error bars indicate standard errors of the means of three repetitions. Different letters above the bars indicate significant difference according to Duncan's multiple range test ($P = 0.05$).

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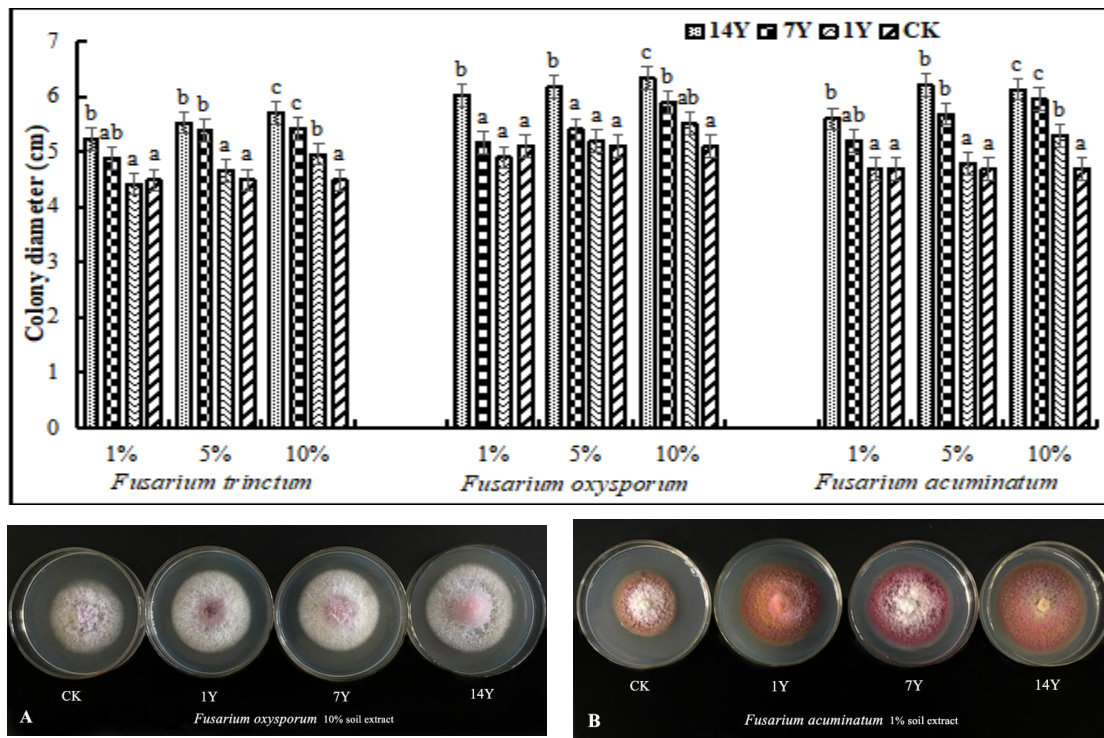
Fig. 5. Effect of rhizosphere soil extracts (final concentration V/V: 1%, 5% and 10%) from fields with alfalfa continuous cropping for 1, 7, and 14 years on mycelial growth of *Fuasrium oxysporum*, *F. trinctum* and *F. acuminatum* that cause alfalfa root rot. A) Effect of 10% soil extract on mycelial growth of *F. oxysporum*; B) Effect of 1% soil extract on mycelial growth of *F. acuminatum.* Error bars indicate standard errors of the means of three repetitions. Different letters above the bars indicate significant difference according to Duncan's multiple range test ($P = 0.05$).

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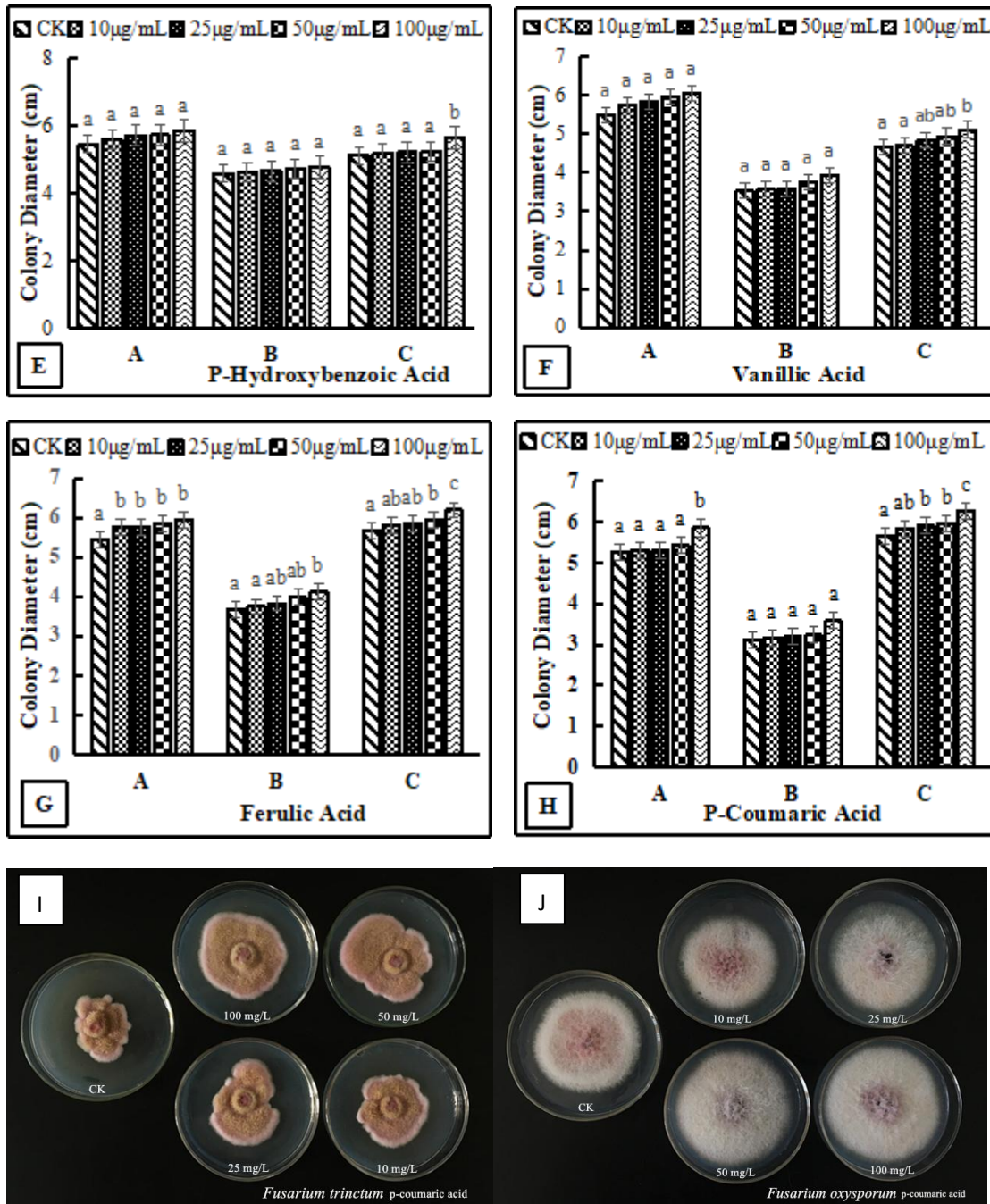
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651 Fig. 6. Effects of different concentrations of phenolic acids on mycelial growth of fungal pathogens that
652 cause alfalfa root rot. A, B and C indicate *Fuasrium oxysporum*, *F. trinctum* and *F. acuminatum*,
653 respectively (E, F, G, H). Error bars indicate standard errors of the means of three repetitions. Different
654 letters above the bars indicate significant difference according to Duncan's multiple range test ($P = 0.05$).
655 I) Effect of p-coumaric acid on mycelial growth of *F. trinctum*; J) Effect of p-coumaric acid on mycelial
656 growth of *F. oxysporum*.

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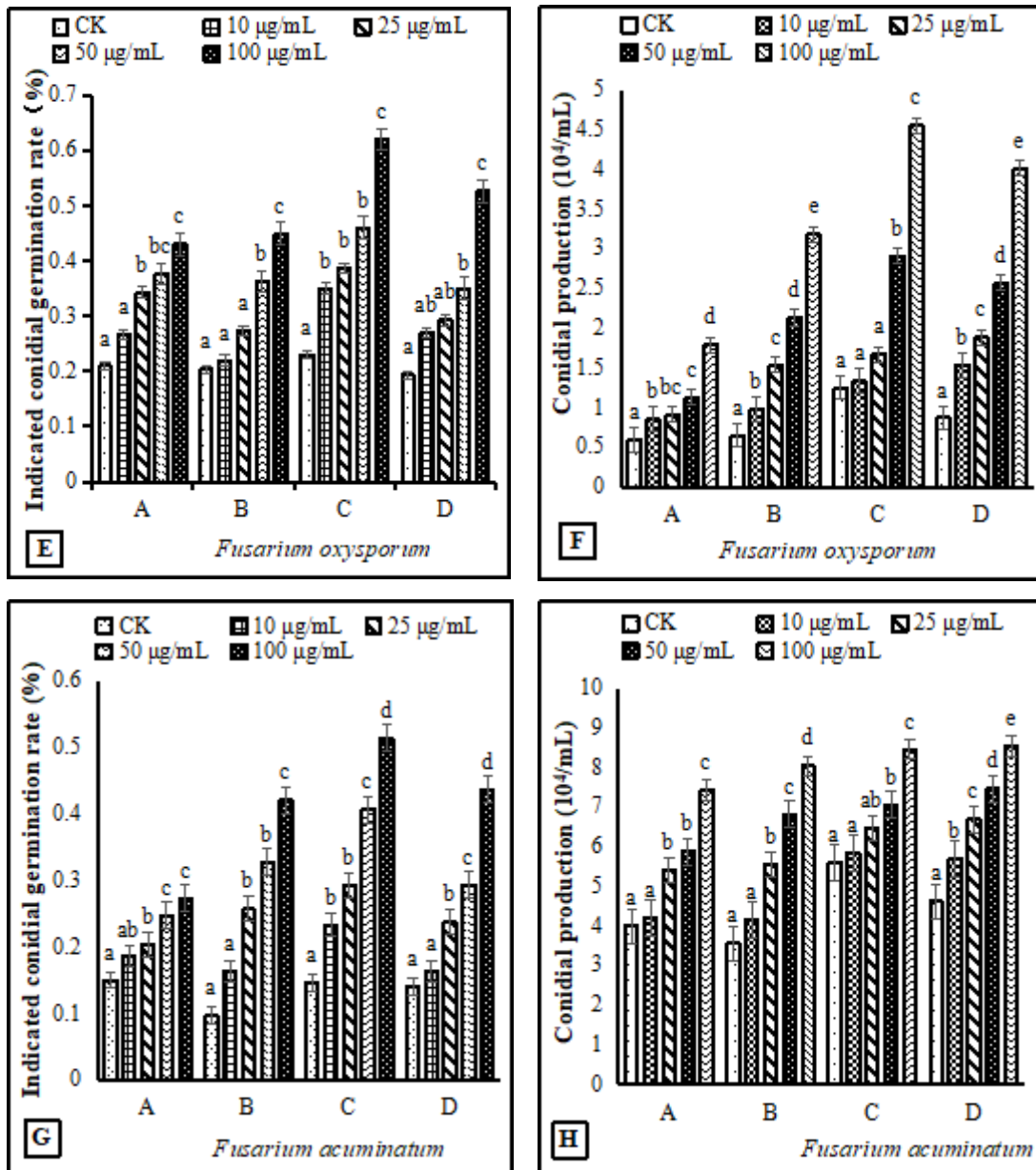
Fig. 7. Effects of phenolic acids on conidial germination and production of *Fusarium oxysporum* (E, F) and *F. acuminatum* (G, H) that cause alfalfa root rot. A, B, C and D indicate p-hydroxybenzoic acid, vanillic acid, ferulic acid and p-coumaric acid, respectively. Different letters above the bars indicate significant difference according to Duncan's multiple range test ($P = 0.05$).

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Fig. 8.   Effect of p-coumaric acid on alfalfa seedling and root rot. A) Inoculated with water as control; B) Inoculated with mixed spore suspensions of *Fusarium trinctum*, *F. acuminatum* and *F. oxysporum*; C) Inoculated with mixed spore suspensions of the three *Fusarium* spp. and 10 μg/mL p-coumaric acid; D Inoculated with mixed spore suspensions of the three *Fusarium* spp. and 50 μg/mL p-coumaric acid.

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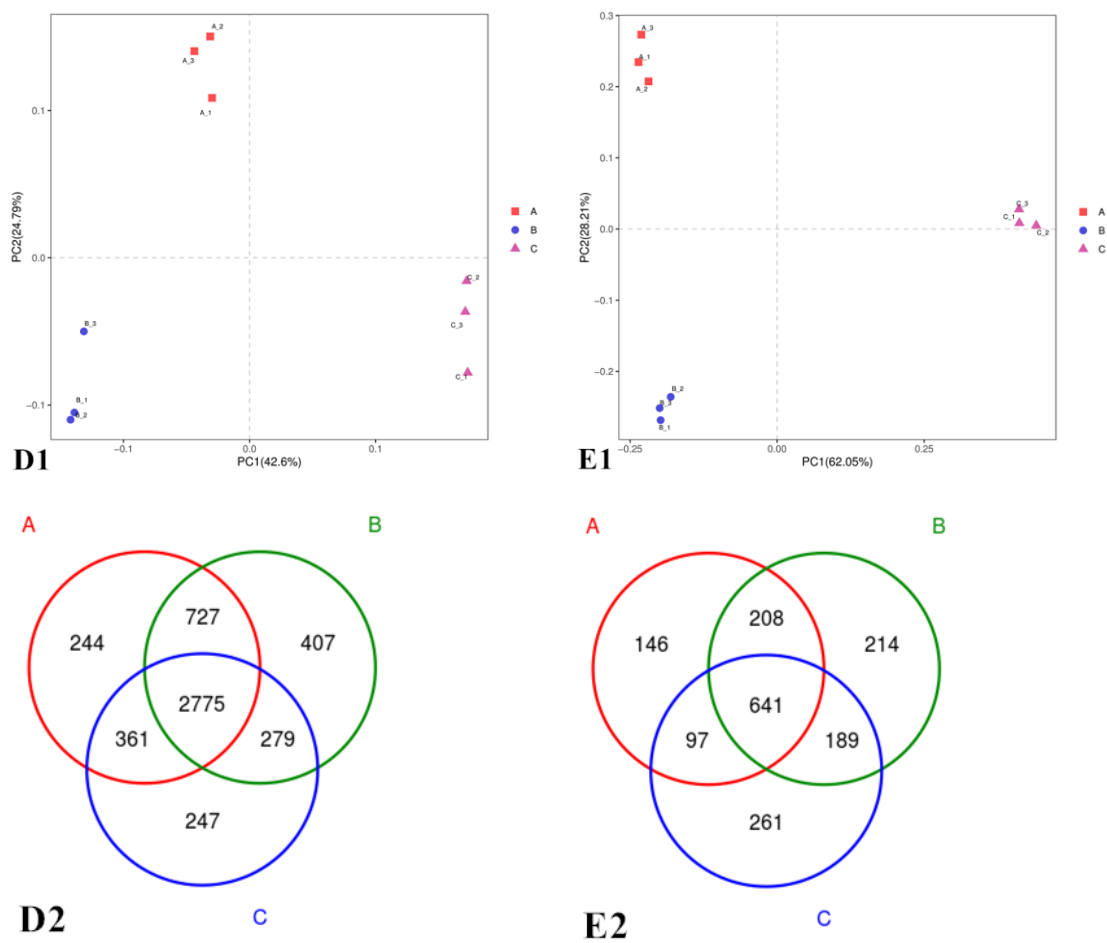
Fig. 9. Principal coordinate analysis (PCoA) showing the similarity of rhizosphere microbial community in fields with continuous cropping of alfalfa for 1 year (A), 7 years (B), and 14 years (C). D1) bacteria, and E1) fungi. D2) Venn diagram of bacteria, and E2) Venn diagram of fungi.

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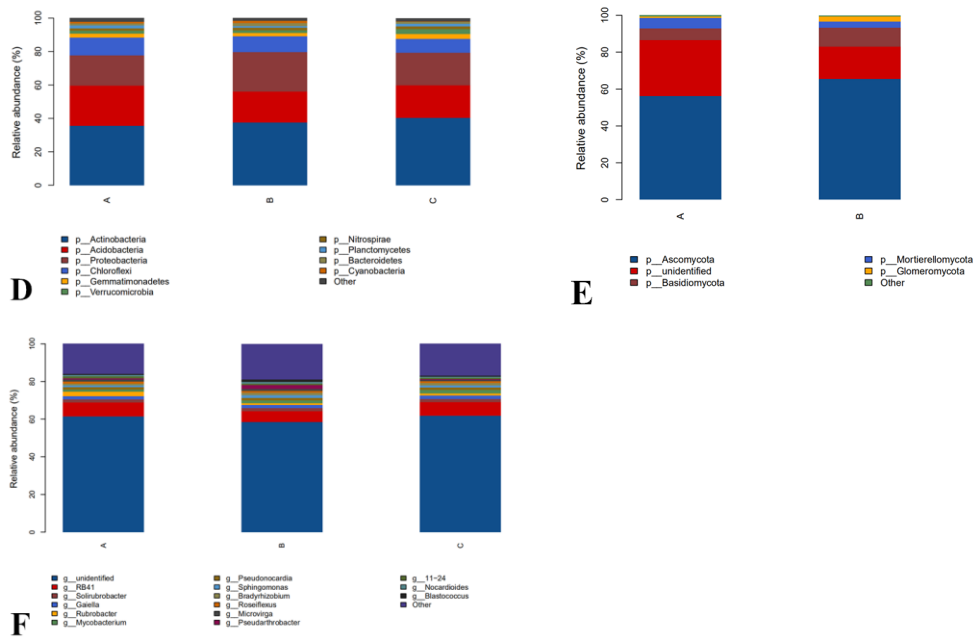
680 Fig. 10. The relative abundance (%) of major bacteria (D) and fungi (E) phylum, and bacterial genus (F),

681 in the microbial community of alfalfa rhizosphere soils from fields with continuous cropping of alfalfa for

682 1, 7, and 14 years.

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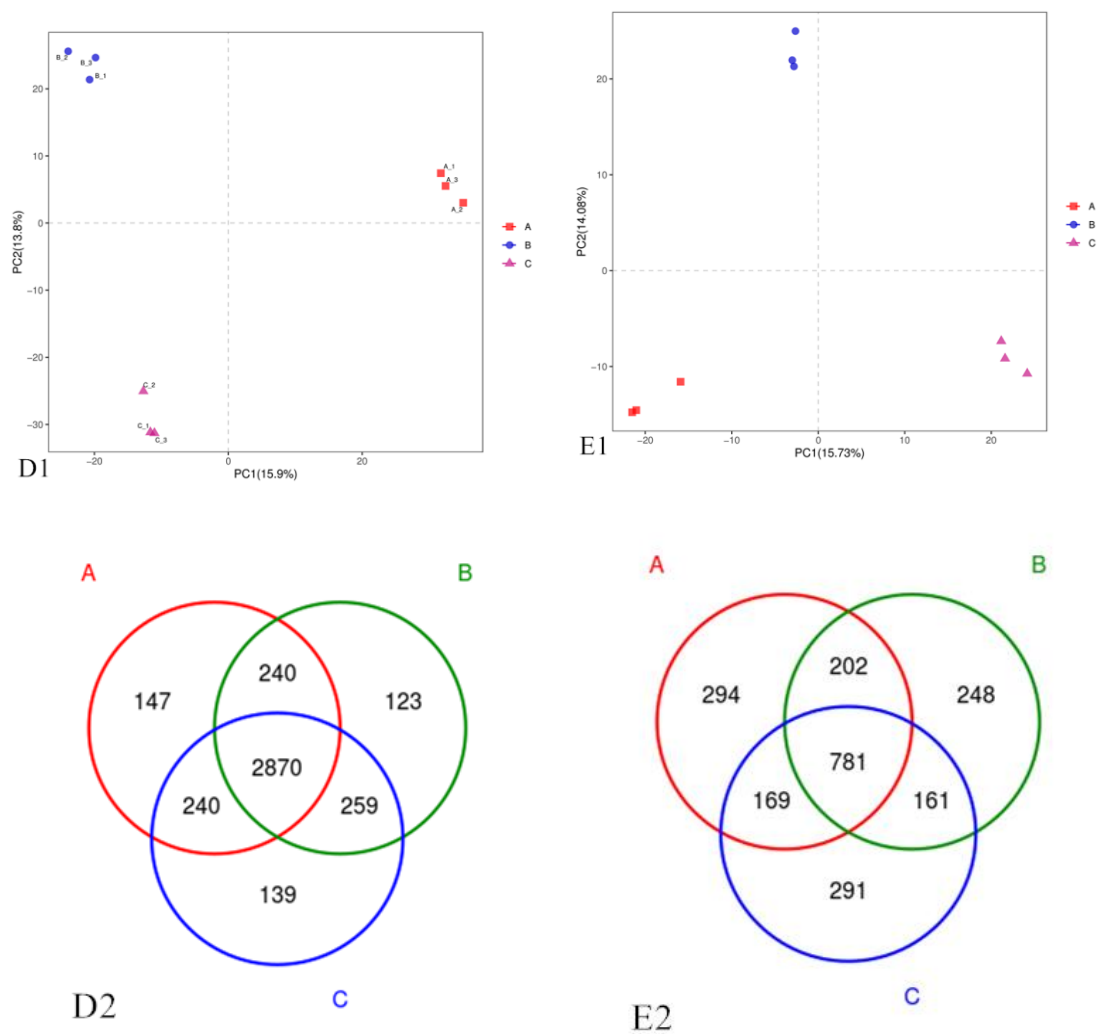
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686 Fig. 11. Principal coordinate analysis (PCoA) showing the similarity of rhizosphere microbial community

687 in fields with A (nontreated), B (treatment with 10 μg/mL p-coumaric acid), C (treatment with 50 μg/mL

688 p-coumaric acid). D1) bacteria, and E1) fungi. D2) Venn diagram of bacteria, and E2) Venn diagram of

689 fungi.

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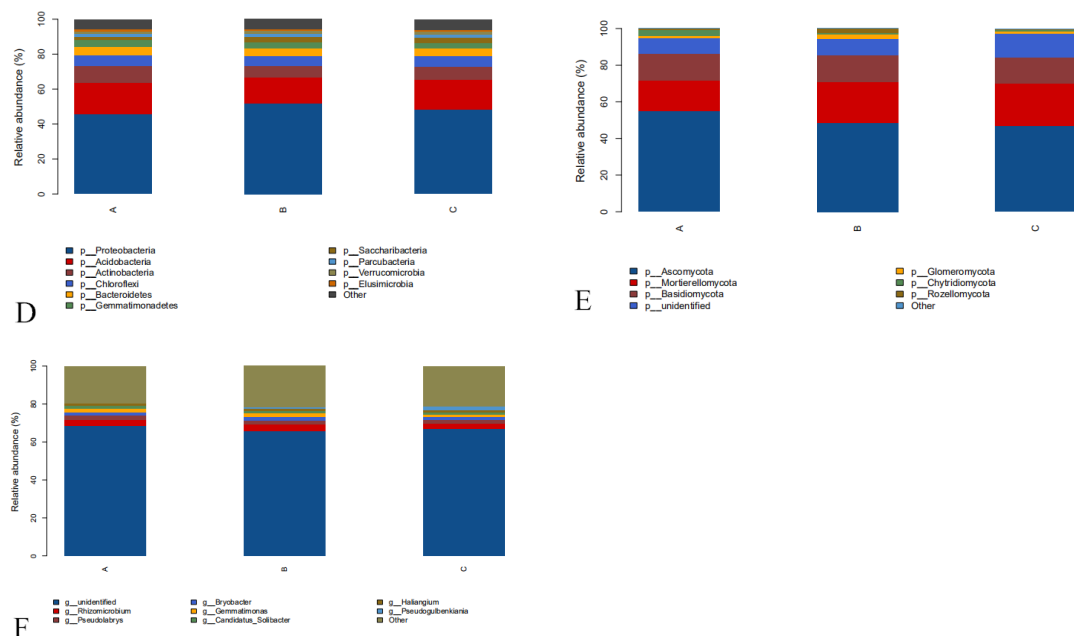
Fig. 12. The relative abundance (%) of major bacteria (D) and fungi (E) phylum, and bacterial genus (F), in the microbial community of alfalfa rhizosphere soils from fields with A) nontreated, B) treatment with 10 μg/mL p-coumaric acid, and C) treatment with 50 μg/mL p-coumaric acid.

Table 1. Nutrient content of rhizosphere soils from fields with alfalfa continuous cropping

CC year[a]	Alkali-hydrolyzed N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)	Available B (mg/kg)	Available Fe (mg/kg)	Available Mn (mg/kg)	Available Cu (mg/kg)	Available Zn (mg/kg)	Available Mg (mg/kg)
14	75.5	2.2	102	0.46	11.2	3.86	1	1.5	139
7	101	3.1	97.1	0.56	8.2	2.9	0.71	0.88	122
1	147.6	3.1	159	0.9	34.2	7.56	0.58	0.85	191

[a] Indicates alfalfa continuous cropping (CC) for 1, 7, and 14 years.

Table 2. Relative content of phenolic acids from rhizosphere soils in fields with alfalfa continuous cropping

CC year[a]	Vanillic acid[b]	p-Hydroxybenzoic acid[b]	Ferulic acid[b]	p-Coumaric acid[b]
1	0.050 ± 0.008 a	0.127 ± 0.014 a	0.031 ± 0.004 a	0.056 ± 0.011 a
7	0.068 ± 0.007 b	0.171 ± 0.013 b	0.026 ± 0.004 a	0.079 ± 0.009 b
14	0.070 ± 0.006 b	0.212 ± 0.017 c	0.046 ± 0.012 b	0.097 ± 0.011 c

[a] Indicates alfalfa continuous cropping (CC) for 1, 7, and 14 years.
[b] Different letters in the column indicate significant difference according to Duncan's multiple range test ($P = 0.05$).