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• LETTER TO THE EDITOR •

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Antifungal activity and phytochemical investigation of the asexual endophyte of *Epichloë* sp. from *Festuca sinensis*

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Dear Editor,

Much research is now being conducted on grasses-*Epichloë* associations. The asexual *Epichloë* species have attracted much attention over the past 30 years as they can provide benefits to important forage grasses, in particular perennial ryegrass (*Lolium perenne*), annual ryegrass (*L. multiflorum*), tall fescue (*Festuca arundinacea*), and meadow fescue (*F. pratense*) [1]. Some findings suggest that grasses with an epichloë endophyte (E+) are more resistant to pathogenic fungi than uninfected (E–) grass [1]. Tests using detached and attached leaves have shown that the size of lesions caused by pathogenic fungi are smaller on E+ leaves than on E– leaves [2]. Products inhibitory to plant pathogenic fungi are produced by epichloë endophytes growing saprotrophically [3,4].

Festuca sinensis is an important cool-season grass species grazed by cattle and sheep and is of increasing importance in managed pastures, especially in cool and semi-arid regions of China. This grass species is frequently host to an asexual symptomless *Epichloë* sp. Recent results demonstrate that *Epichloë* increases the seed germination and seedling growth of *F. sinensis* [5]. Little is known of the bioactivity of secondary metabolites of this endophyte and thus targeted research is required.

Our aim of this study was to further assess the role of endophytes of grasses on disease development in host grasses. To determine if these products could affect the growth of fungal pathogens, dual culture studies were used along with an examination of lesion development on leaves of E+ and E– grasses [3]. Also reported in this paper is the range of chemicals that were identified using gas chromatography-mass spectrometry (GC-MS) in extracts of liquid culture of the endophyte that had antifungal activity in *in vitro* tests.

Lesions developed on both E+ and E– leaves inoculated with all four fungi (spore concentrations, 10^5 cfu mL^{-1}) while the control E+ and E– leaves remained lesion-free (Figure 1A–H). The disease incidence and lesion length increased as time passed after inoculation. The disease incidence on E+ leaves caused by *Alternaria alternata*, *Fusarium acuminatum*, *Bipolaris sorokiniana*, and *Curvularia lunata* was significantly less than those on E– leaves on the 7th day after inoculation (P<0.05; Figure 1A–D). The lesion length caused by *F. acuminatum*, *B. sorokiniana*, and *C. lunata* was significantly smaller on E+ leaves than on E– ones after 3–7 d, 4–7 d, and 4–7 d, respectively (P<0.05; Figure 1F–H). *A. alternata* gave very little symptom

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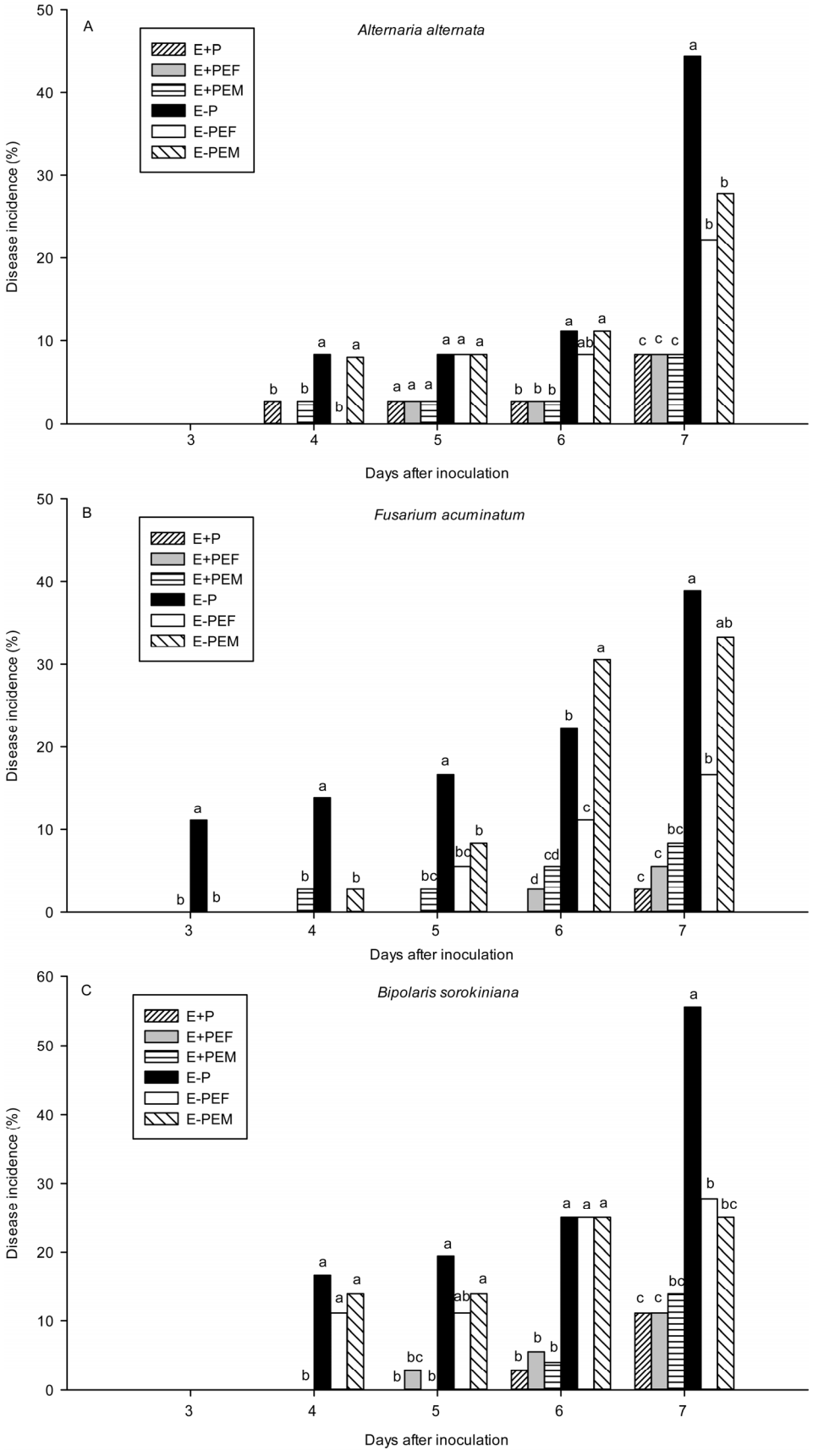

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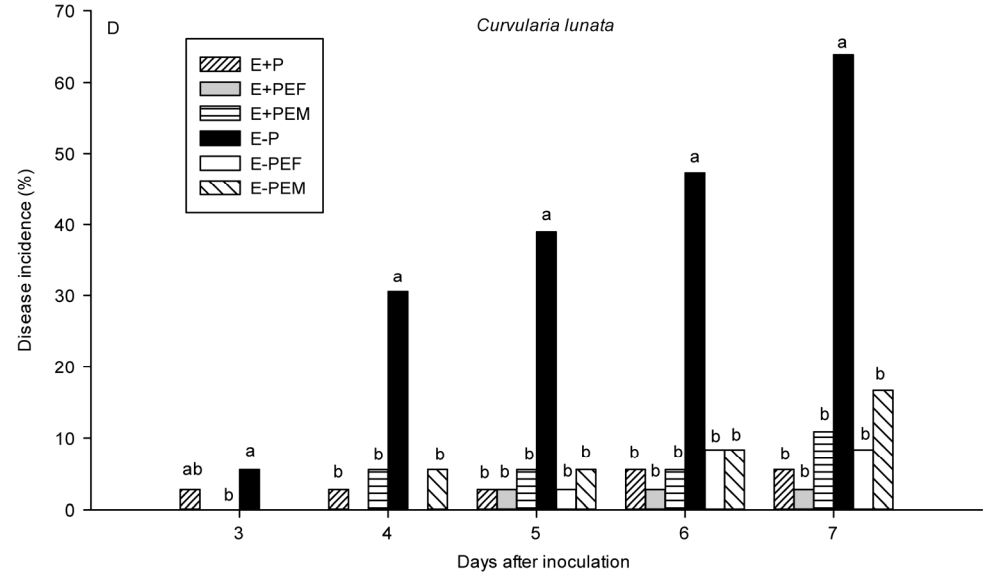

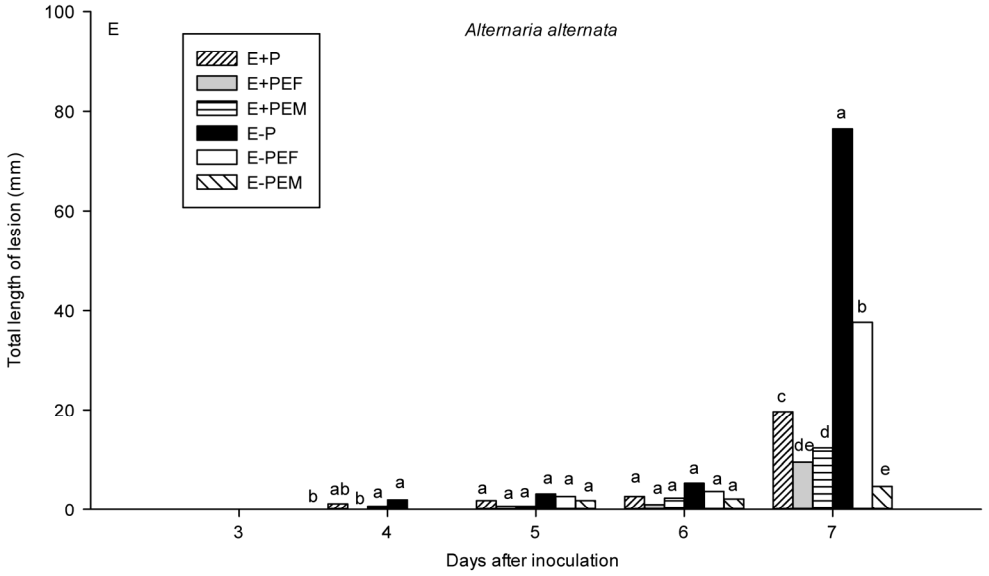

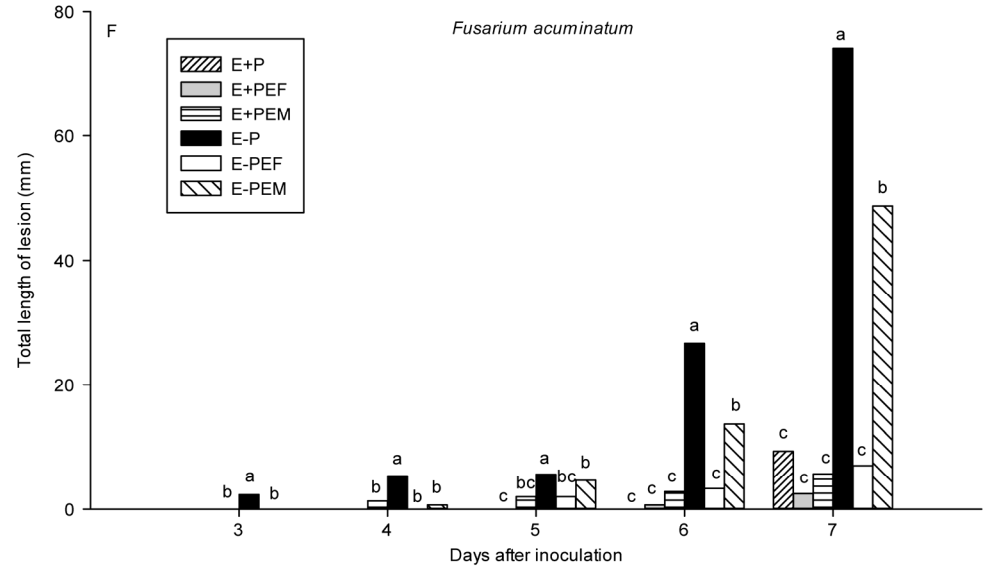

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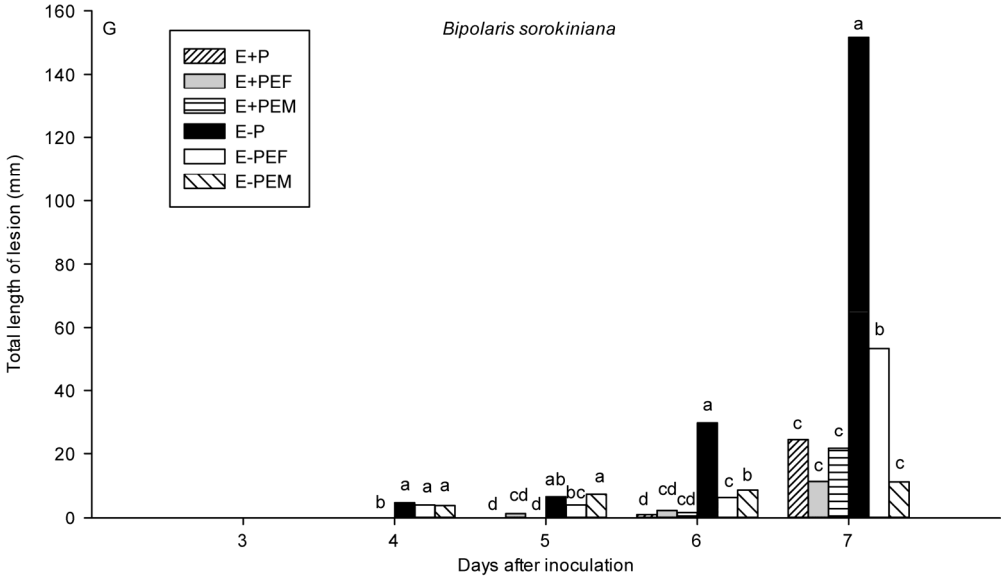

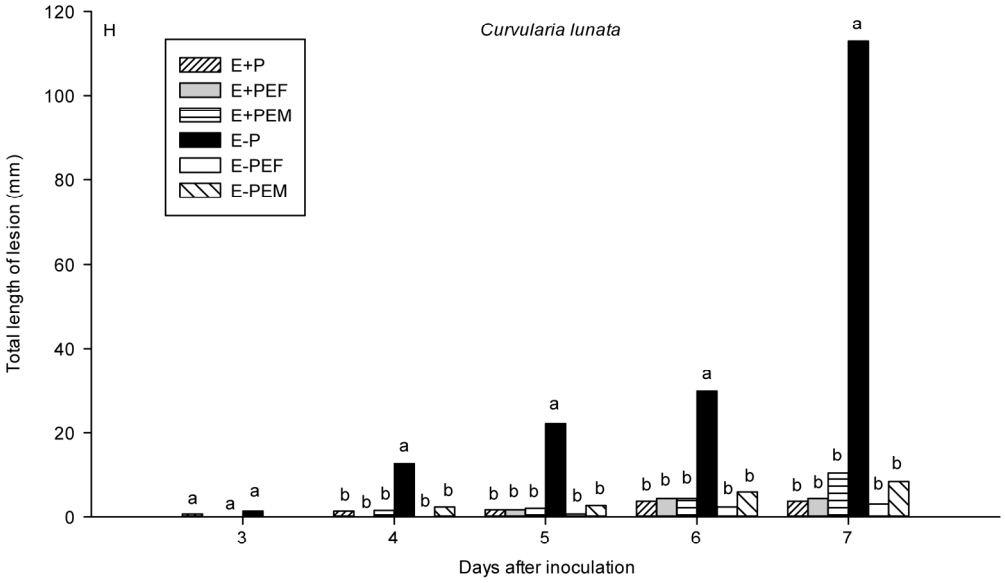

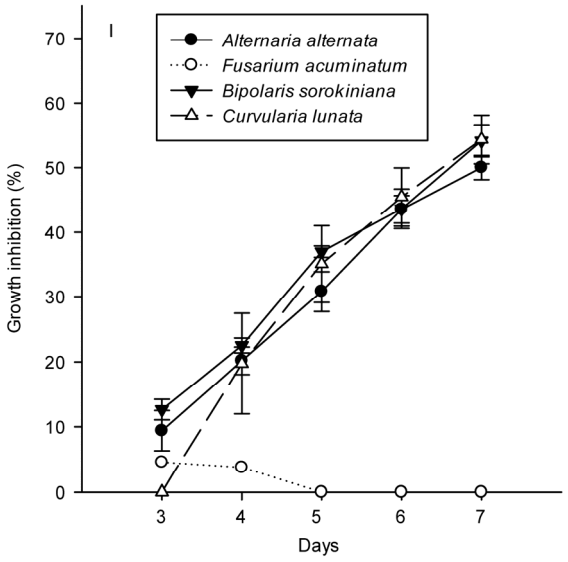

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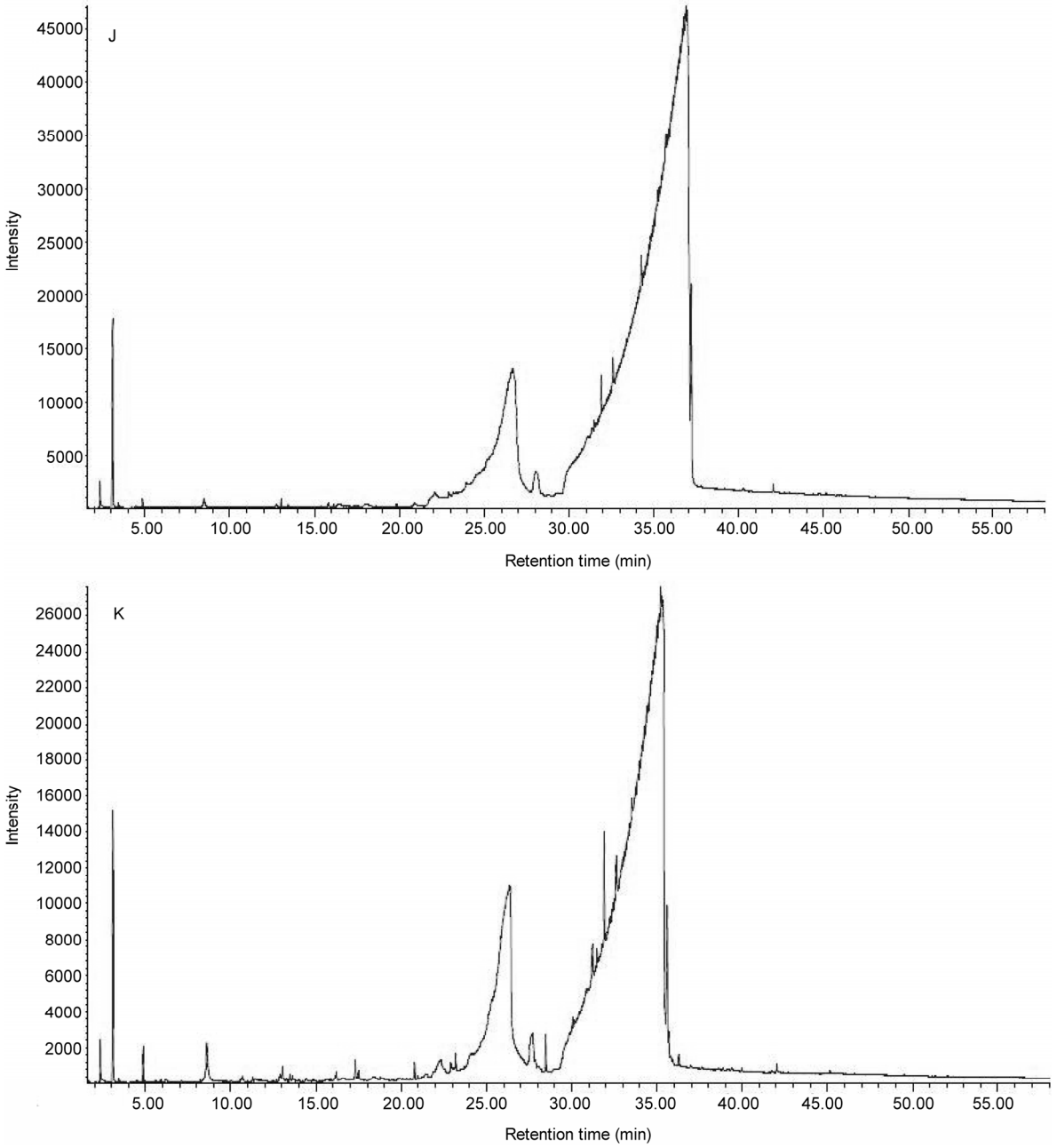

Figure 1 Inhibitory effects of *Epichloë* sp. on the plant pathogenic fungi, *Alternaria alternata*, *Fusarium acuminatum*, *Bipolaris sorokiniana*, and *Curvularia lunata.* A–H, Effects of endophyte and/or inhibitory substances on disease development on detached leaves of *F. sinensis.* Values are means of three replicates. Non-matching small letters at the same day indicate a significant difference among treatments (LSD, $P<0.05$). E-P, E-PEF, and E-PEM represent each E– leaf inoculated with spore suspensions from each fungal pathogen, the mixtures with spore suspensions from each fungal pathogen and inhibitory substances EF, and the mixtures with spore suspensions from each fungal pathogen and inhibitory substances EM, respectively. E+P, E+PEF, and E+PEM represent each E+ leaf inoculated with spore suspensions from each fungal pathogen, the mixtures with spore suspensions from each fungal pathogen and inhibitory substances EF, and the mixtures with spore suspensions from each fungal pathogen and inhibitory substances EM, respectively. EM and EF represent ethyl acetate extracts from mycelium and fermentation broth of the *Epichloë* sp. XH03, respectively. I, Co-culture between *Epichloë* sp. XH03 and the plant pathogenic fungi. J and K, Total ion chromatogram of the ethyl acetate extracts from mycelium (J) and fermentation broth (K) of the *Epichloë* sp. XH03.

development until day 7 and then the length of the lesions on the E– leaves greatly lengthened. The lesions on the E+ also lengthened but to a much smaller amount ($P<0.05$; Figure 1E). This rapid increase in lesion length on the 7th days was also observed with E– leaves infected with *B. sorokiniana*.

Using the dual culture procedure an inhibitory effect was observed with the isolates of *Epichloë* sp. XH03 on the

growth of *A. alternata*, *B. sorokiniana*, and *C. lunata* between the 3rd and the 7th day. The inhibition rate for each fungus was 9.38%–50.00%, 12.67%–54.13%, and 0.02%–54.35%, respectively (Figure 1I). The growth inhibition of *A. alternata*, *B. sorokiniana*, and *C. lunata* increased to above 50% on the 7th day. There was slight inhibition of *F. acuminatum* on the 3–4th day but no inhibitory effects after 5–7 days.

The disease incidence observed on E+ leaves inoculated with conidia in solutions of inhibitory substances (2.00 mg mL^{-1}, EF or EM, ethyl acetate extracts from fermentation broth or mycelium of the *Epichloë* sp. XH03) showed no difference compared to E+ leaves inoculated with conidia in water (Figure 1A–H). The length of lesions on E+ leaves for three of the four pathogens inoculated as conidia in solutions of inhibitory substances also did not differ from those inoculated in water. However, the lesion length caused by *A. alternata* conidia in water on the E+ leaves were significantly longer than on the ones inoculated with the mixture of *A. alternata* spore suspension and inhibitory substances EF.

The disease incidence on the E– leaves inoculated with *A. alternata* or *F. acuminatum* conidia in water was significantly higher than on the E– leaves inoculated with conidia in inhibitory substances EF on the 7th day ($P<0.05$; Figure 1A and B). Moreover, on the E– leaves the disease incidence caused by *B. sorokiniana* or *C. lunata* was also significantly higher than on the E– ones inoculated with inhibitory substances EF or EM on the 7th day ($P<0.05$; Figure 1C and D). The lesion length caused by the pathogenic fungi *A. alternata*, *F. acuminatum*, *B. sorokiniana*, or *C. lunata* on the E– leaves were greatly longer than on the E– ones inoculated with each of the fungi with inhibitory substances EF or EM after 7 d, 3–7 d, 6–7 d, or 4–7 d, respectively ($P<0.05$; Figure 1E–H).

The analysis of ethyl acetate extracts was carried out by GC-MS (6890N-5975C, Agilent Technologies, USA). Chromatograms and mass spectra were evaluated and compounds determined using a library from the NIST 08 database (Figure 1J and K). Seven compounds were identified from mycelium while 17 compounds were identified from fermentation broth. The major chemical constituents that were found in the mycelial extract EM are D-mannitol (56.25%), hexadecanoic acid (3.39%), phenol, 2,2′-methylenebis[6-(1,1-dimethylethyl)-4-methyl]- (0.81%), and 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester (0.47%). The major chemical compositions in the fermentation supernatant EF were determined as D-mannitol (29.21%), indoleacetic acid (1.53%), 9,12-octadecadienoic acid (Z,Z)- (1.23%), pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- (1.08%), benzeneethanol, 4-hydroxy- (1.17%), 1H-indole-3-ethanol (1.07%), cis-13-octadecenoic acid (0.90%), and benzeneacetic acid (0.72%).

In conclusion, the *Epichloë* sp. present in the *F. sinensis* plants that we studied has inhibitory effects on pathogenic fungi, both when the fungus is growing saprotrophically in culture and biotrophically in the plants. Some of the products obtained from saprotrophic growth may have potential as useful antifungals. It is thus possible that the use of selected strains of *Epichloë* sp. that produce antifungal products may lead to the formation of novel *F. sinensis* associations that have enhanced resistance to pathogenic fungi.

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