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Table: 1, Figs : 5

Allelopathic effects of allelochemicals of *Ginkgo biloba* leaf on *fusarium* wilt (*Fusarium oxysporum*) of hot pepper

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ABSTRACT

In laboratory bioassay, we analyzed the allelochemicals in leaves of ginkgo (*Ginkgo biloba*) and determined their allelopathic effects on fusarium wilt (*Fusarium oxysporum*) of hot pepper. The extract of *G. biloba* leaves inhibited the growth of fungus at 0.04 g l^{-1} concentrations, whereas, control was stimulatory. Twenty two types of allelochemicals were identified and 8 (2-Hydroxypropionic acid, benzoic acid, myristic acid, 3-hydroxybenzoic acid, 3,4-dihydroxy cinnamic acid, n-hexadecanoic acid, 8-octadecenoic acid, eicosanoic acid. 2-hydroxypropionic acid) were bioassayed for their allelopathic effects on mycelial growth and spore germination of fusarium wilt. The myristic acid, 9-octadecenoic acid and eicosanoic acid at 200 µM concentrations, promoted the mycelial growth and spore germination of fusarium wilt. However, 2-hydroxypropionic acid, benzoic acid, 3-hydroxybenzoic acid, 3,4-dihydroxy-cinnamic acid and n-Hexadecanoic acid inhibited the mycelial growth and spore germination of fusarium wilt at this concentration. The allelochemicals differed in effects on fusarium wilt.

Keywords: Allelochemical, allelopathy, *Capsicum frutescens*, *Fusarium oxysporum*, GC-MS, *Ginkgo biloba*, inhibition, leaf, mycelial growth, promotion, spore germination

INTRODUCTION

Hot pepper (*Capsicum frutescens*) is an important economic crop, and its fruit has high nutritional value due to many antioxidant compounds [phenolics, vitamin C and carotenoids (41)]. Since the 1990s, its production is increasing rapidly worldwide, due to increased consumption, breakthrough in its breeding technology and culture leading to year-round cultivation (12,39). It is commercially cultivated in China, Korea, East Indies, USA and several other countries (34,35) and annual production in China is approx 215,000 t (35). However, the soil sickness problems associated with its continuous cropping have become a key topic of research, the disease becomes more serious with the increase in number of years of continuous cropping, severely limiting its cultivation. The hot pepper yields decreases by 10 to 15%, 20 to 30% and 30 to 50% with continuous cropping for 1-year, 2-years and 3-years, respectively (40). The researcher have found that one of the main causes of continuous cropping problems consist of the accumulation of harmful

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microbes. Although intercropping, rotation, bio-control, use of resistant varieties, grafting and the addition of allelochemicals and organic fertilizer have been used to overcome the problem but some of these remedies are not feasible. The addition of allelochemicals to soils has been much studied to prevent the attack of pathogens and promote the crop growth (11,17,31).

Recently Ginkgo biloba extracts have attracted the international attention as its leaves contains flavonoids and phenolic acids with anti-oxidative, anti-ageing and other pharmacological effects (20). The chemical composition and pharmacological effects of the extracts from G. biloba leaves have been sudied (32). The extracts of *G. biloba* were tested against *Escherichia coli, Pseudomona aeruginosa, Klebsiella pneumoniae, Bacillus subtilis, Enterococcus* spp. and *Staphylococcus aureus* by using agar well diffusion (6). The leaf extracts of *G. biloba* are antimicrobial against all 3-groups of microorganisms, bacteria being most sensitive, followed by actinomycetes and fungi (24). The exopleura of Ginkgo biloba had compounds of 2-hydroxy-6-(8-pentadecenyl) and 2-hydroxy-6-(10-heptadecenyl) salicylic acid (3). The extracts of G. biloba are thermal stable, as temperature did not influence its antibacterial effects inhibitory, its antibacterial effects varied with extraction methods, strains and extract concentrations. This study aimed,. to analyzed the allelochemicals of G. biloba leaves, determine their alleopathic effects on fusarium wilt (*Fusarium oxysporum*) of hot pepper and, to know the antiviral mechanisms of these allelochemicals.

MATERIALS AND METHODS

I. Extract preparation

Dried leaves of *G. biloba* were collected from the campus of Shenyang University in Autumn. These were ground using a pulveriser (type, FZ102; voltage, 220 V; power, 0.32 kW) and 1 g *G. biloba* leaves were extracted with ether and n-hexane (1ml:1 ml ratio). The mixture was gently shaken for 2 h at 15^{o}C-20 ^{o}C with an electric shaker (20 rpm). The extracts were combined and filtered through Whatman (No. 6) filter paper. The filtrates were concentrated in a rotary vacuum evaporator at 40 ^{o}C and then was sterilised by filtration with 0.22 μm filters. Concentrations of the extracts of *G. biloba* leaves were prepared to determine their allelochemicals and bioassayed the effects on mycelium growth and spore germination.

II. *Fusarium oxysporum* preparation

Before the test, the fungus *Fusarium oxysporum* was isolated from the infected hot pepper plants growing in an infested seedling nursery area and verified by a pathology laboratory according to Booth's postulate (2).

III. GC-MS analysis of *Ginkgo biloba* leaf allelochemicals

The concentrated fractions extracted from *G. biloba* leaves were analyzed using a gas chromatograph coupled to a mass spectrometer (GC-MS, Hitachi M-80B, Hitachi, Tokyo, Japan) before or after methylation with diazomethane from N-methyl-N-nitoso-

p-toluene sulfonamide. An aliquot of each concentrated fraction (1 or 2 ml) was diluted in 50 ml ether, treated with diazomethane and concentrated to 5 µl in a rotary evaporator then in N_2 stream in a water bath at 35 ^oC. One µl of the concentrated sample was injected into a GC-MS with a capillary column (0.25 mm×60 m) of TC-5 (GL Science, Tokyo, Japan). Helium was used as the carrier gas at a pressure of 78.4 kN m^{-2}. The column was held initially at 100 ^oC for 2 min and then raised at 5 ^oC min^{-1} to a final temperature of 260 ^oC for 10 min. The injector temperature was held at 270 ^oC. The ionization voltage and temperature in the electron impact (EI) mode were 70 eV and 250 ^oC, respectively.

IV. Mycelial growth

(i) *Ginkgo biloba* leaves allelopathy

The mycelial growth of *F. oxysporum* was measured by adopting the growth rate method. Five ml of 0.04 g l^{-1} extract was added to 5 ml of thawed culture medium sterilized and cooled to 40°C in slab form. Each treatment was five replicated five times, with pure Potato Dextrose Agar (PDA) medium as control. A 6 mm- dia pure culture *F. oxysporum* disc was inoculated onto the medium and incubated at 25°C in darkness. The colony diameter was measured with cross method, 3 d after incubation and the inhibition rate was calculated.

(ii) *Ginkgo biloba* leaves allelochemicals' allelopathy

The method was same as above. Different allelochemicals of *G. biloba* leaves were used as experimental materials at 200 µM concentration.

V. Spore germination

(i) *Ginkgo biloba* leaves allelopathy

The effects of extract on spore germination were measured by the suspension method (4). The spore suspension was prepared by placing the *F. oxysporum* in 50 ml beaker and added 0.1 ml distilled water. Before mixing well, the spore suspension was filtered into a microcentrifuge tube and observed with microscope. During the observation, the concentration of spores was diluted to ensure that 100 spores could be observed with a 600x binocular microscope. A 10 µl aliquot of *G. Biloba* leaves extract was mixed with 10 µl suspension on a slide. The slide was incubated at 25°C and observed using a microscope after 7, 9 and 11 h. The numbers of germinated spores and total spores were recorded to calculate the germination rate. Each treatment was replicated thrice and ether was used as control. Each replication was observed with ten fields of view.

(ii) *Ginkgo biloba* allelochemicals' allelopathy

The method was same as above. Different allelochemicals of *G. biloba* leaves were used as experimental materials at 200 µM concentration.

VI. Response index (RI):

The results were expressed in RI values as per the Williamson method (34), as under:

If $T \geq C$, then $RI = 1 - C/T$;

If $T < C$, then $RI = C/T - 1$

Where, C: Control data, T: Treatment data and RI : Allelopathic index. The

intensity of the effect was expressed in RI value. RI value > 0 indicates stimulation, whereas, RI < 0 indicates inhibition.

The original data was processed by DPS software.

VII. Statistical analysis

The experimental data was processed with excel software and analyzed with analysis of variance using DPS version 7.05 (30). Prior to anova with multiple-comparison tests, variance ratio statistics were tested for the treatments significance. The multiple-comparison tests include least significant difference (LSD) and least significant ranges (LSR) and LSR includes q test and Duncan's method of LSR was used in this paper. And the significant differences of the RI of hot pepper mycelium growth and spore germination of *F. oxysporum* treated by Gingko leaf were tested. The small and capital letters indicate the difference in 5 and 1% level respectively by Duncan's method of LSR test.

RESULTS AND DISCUSSION

***Ginkgo biloba* leaves extract against *Fusarium* wilt**

(i) Mycelial growth : The RI of treatments with hot pepper leaves were > zero, and the RI of treatments with *Ginkgo biloba* leaves were all < zero (Fig. 1). It represented that hot pepper plants are very susceptible to fusarium wilt due to the accumulation of pathogens in the soil of continuous cropping of hot pepper. The *Ginkgo biloba* leaves *in-vitro* were inhibitory to the mycelial growth of *F. oxysporum*. The RI decreased and then rose slightly i.e. the inhibition decreased after an initial increase, with increase in processing time. In contrast, control (the extract of hot pepper leaves) had stimulatory effect. There were no significant differences among the control treatments at 4, 5, 6 and 7 d. The Gingko leaf treatment exhibited highly significant differences from control. In general, the Gingko leaves were inhibitory at 0.04 g l^{-1} concentration. There was no positive correlation between the *F. oxysporum* biomass and culture time. For *G. biloba* leaves extracts at 0.04 g l^{-1} did not affect the mycelial growth at 4, 5, 6 d. The inhibitory affects of extracts of *G. biloba* leaves at 4, 5, 6 d on mycelial growth were drastic than at 7 d ($P < 0.01$). The magnitude of inhibitions of the extracts of *G. biloba* leaves at 3 d were significantly lower than at 7 d ($P < 0.01$).

(ii) Spore germination : Extracts of *G. biloba* and Control inhibited the spore germination of *F. oxysporum* at 0.04 g l^{-1} and the inhibitory effect was inversely proportional to the culture time (Figure 2). The RI of all treatments were < zero and the value of RI increased with time. The extract of *G. biloba* inhibition to germination of *F. oxysporum* spores was stronger than control. The inhibition of spore germination with extract of *G. biloba* was significantly higher than control after 7 h ($P < 0.01$). The inhibitory effects of extract of *G. biloba* at 9 and 11 h on spore germination were smaller than extract of *G. biloba* at 7 h. The differences in

inhibition of extract of *G. biloba* at 9 and 11 h were not significant. The culture duration affected the spore germination inhi bition with the extract of *G. biloba* b and control similarly in the order: 7 >9 >11 h.

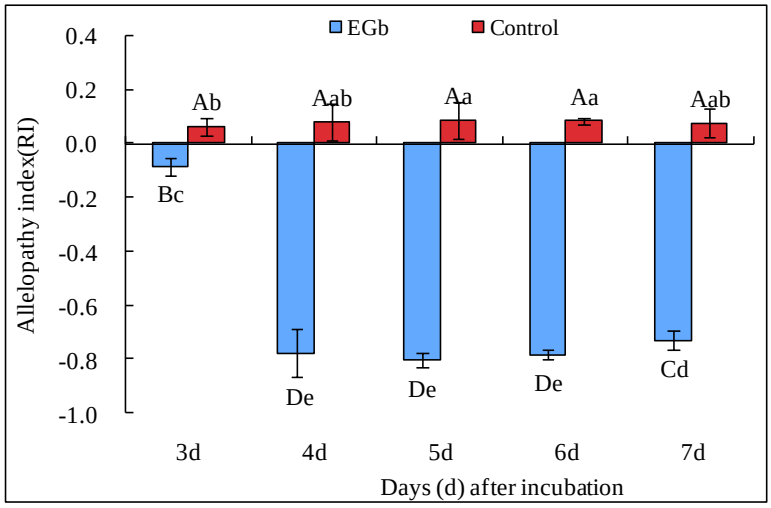

Figure 1. Effects of gingko leaf extract on mycelial growth of Fusarium wilt-causing fungus EGb, The extract of *G. biloba* (0.04 g l^{-1}); Control, The extract of hot pepper leaves (0.04 g l^{-1}). The small and capital English letters indicate the difference at 5% and 1% level, respectively, by LSR test.

Researches have shown that the main causes of continuous cropping are the accumulation of harmful microbes, changes of soil microorganisms, salinization of secondary soil and auto-toxicity of vegetable crops (27,29). The organic fertilizer as gingko leaves added in soil improved the soil microbial flora and prevented the root diseases caused by the unbalanced soil microflora after continuous cropping (24). Our study also confirmed that the extract of gingko leaves was very inhibitory to both spore germination and mycelial growth of *F. oxysporum* build up by the continuous cropping problem in hot pepper. Zhang *et al*. (38) reported that the extracts of *Cnidium monnieri* and *Sophera flavescens* had dose-dependent inhibitory effects on the mycelial growth of *Verticillium dahliae*, while Wang *et al*. (33) demonstrated the antifungal activity of root exudates against *Verticillium* wilt. Similarly, the incidence of *Fusarium* wilt of cotton and spore germination was decreased by mint volatile compounds (13,14,16).

GC-MS analysis of allelochemicals of *Ginkgo biloba* leaves

The extracts of *G. biloba* leaves were analyzed and numerous compounds were detected (Fig. 3 and Table 1). Twenty-two allelochemicals were found in compounds [2-hydroxypropionic acid, benzoic acid, myristic acid, 3-hydroxybenzoic acid, 3,4-dihydroxy cinnamic acid, n-hexadecanoic acid, 8-octadecenoic acid, eicosanoic acid]. 2-hydroxypropionic acid is soluble in water. Benzoic acid, 3-hydroxybenzoic acid and

3,4-dihydroxy cinnamic acid are only slightly soluble in water. Myristic acid, n-hexadecanoic acid, 8-octadecenoic acid and eicosanoic acid are insoluble in water.

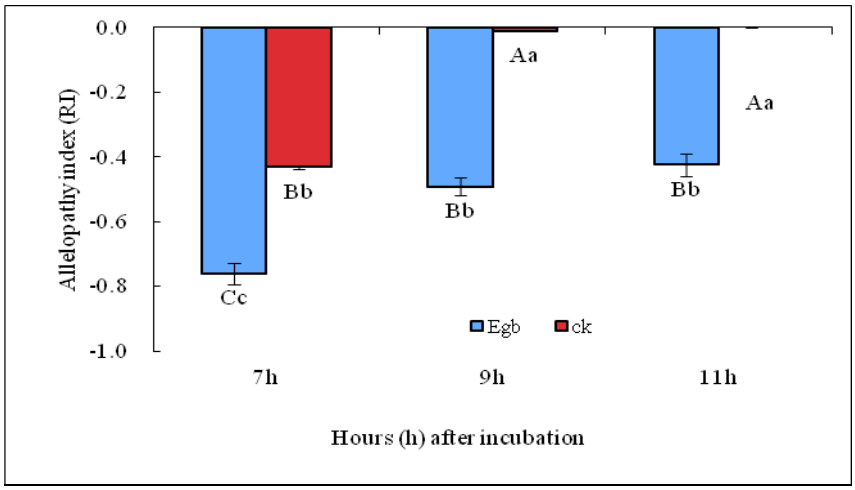

Figure 2. Effects of gingko leaf extract on spore germination of seedling blight-causing fungus EGb, The extract of *G. biloba* (0.04 g l^{-1}); Control, The extract of hot pepper leaves (0.04 g l^{-1}). The small and capital English letters indicate the difference at 5% and 1% level, respectively, by LSR test.

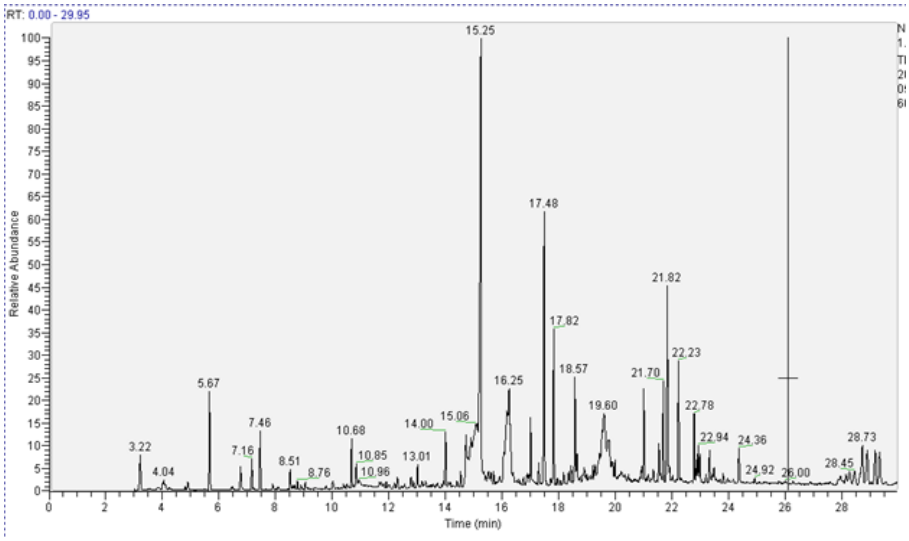

Figure 3. Gas chromatogram of *G. Biloba* leaves

Table 1. The allelochemicals identified in the leaf extracts of *Ginkgo biloba* and used in mycelial growth and spore germination experiment

No.	RT (Min)	Molecular formula	Chemical name	No.	RT (Min)	Molecular formula	Chemical name
1	3.22	$C_3H_6O_3$	2-ydroxypropionic acid	12	17.48	C_7H_8O	Benzyl Alcohol
2	5.67	$C_3H_8O_3$	1,2,3-propanetriol	13	17.82	$C_8H_{10}O_5$	Phenylethyl Alcohol
3	7.16	C_6H_5O	P-hydroxybenzene	14	18.57	$C_{17}H_{34}O_2$	Hexadecanoic acid ,methyl ester
4	7.46	$C_7H_6O_2$	Benzoic acid	15	19.6	$C_{16}H_{32}O_2$	n-Hexadecanoic acid
5	8.51	$C_7H_8O_2$	P-hydroxybenzoic acid	16	21.7	$C_{18}H_{34}O_2$	8-octadecenoic acid
6	10.68	$C_{14}H_{28}O_2$	Myristic acid	17	21.82	$C_{16}H_{32}O_2$	Tetradecane acid, 12-methyl,methyl ester
7	10.85	$C_3H_4O_2$	Acrylic acid	18	22.23	$C_{13}H_{34}O_2$	3,12-Octadecadienoic acid(Z,Z)- methyl ester
8	13.01	$C_7H_6O_3$	3-hydroxybenzoic acid	19	22.78	$C_{27}H_{56}$	Heptacosane
9	14.00	$C_6H_6O_2$	O-diphenol	20	22.94	$C_{19}H_{32}O$	3-tridecylphenol
10	15.25	$C_9H_8O_4$	3,4-dihydroxy-cinnamic acid	21	24.36	$C_{20}H_{40}O_2$	eicosanoic acid
11	16.25	$C_7H_6O_5$	2,4,6-trihy-droxybenzoic acid	22	28.73	$C_{20}H_{30}O_3$	3-pentadecylphenol

RT: Retention Time

With long history of medicinal properties, ginkgo may be a promising candidate as source of novel compounds with allelopathic activity. An allelopathically active substance 2-hydroxy-6-(10-hydroxypentadec-11-enyl) benzoic acid from the leaves of *Ginkgo biloba* inhibited the root and shoot growth of garden cress and timothy (8). The total flavonoids present in gingko leaves proved antimicrobial against the *Staphylococcus aureus*, *Escherichia coli*, *Fusarium graminearum* Schwabe and *Alternaria alternata*.. In our study, we found 22-allelochemicals in extract of leaves, which affects the plant or microorganisms (5,10,25,26,28).

Ginkgo biloba leaves allelochemicals against *Fusarium* wilt

(i) Mycelial growth: The treatments of myristic acid, 9-octadecenoic acid, eicosanoic acid promoted the mycelium growth of *Fusarium* wilt. Their RI value were > zero. While the treatments of 2-hydroxypropionic acid, benzoic acid. The 3-hydroxybenzoic acid, 3,4-dihydroxy-cinnamic acid and n-Hexadecanoic acid inhibited the mycelium growth of *Fusarium* wilt. Their RI values were < zero (Fig. 4). The most effective antibacterial allelochemical of all treatments was 3-hydroxybenzoic acid. The 9-octadecenoic acid and eicosanoic acid showed significant stimulatory activities after 5 d incubation of *Fusarium* wilt, while myristic acid showed significant stimulatory activity after 6 d, but there was no significant change between 6 d and 7 d. When *Fusarium* wilt was incubated for 6-7d, the benzoic acid, 3-hydroxybenzoic acid and 3,4-dihydroxy-cinnamic acid showed highest inhibitory effects on its mycelium growth.

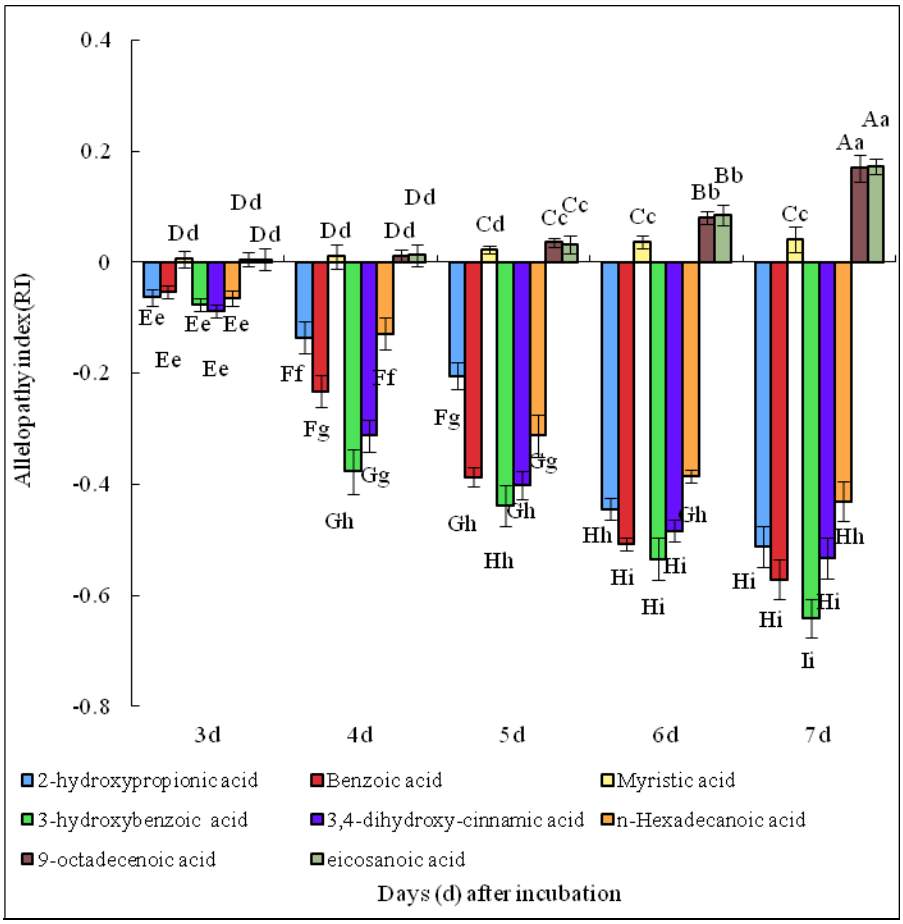

Figure 4. Effects of allelochemicals of gingko leaf extract on mycelial growth of seedling blight-causing fungus

(ii) Spore germination: The effects of allelochemicals of *G. biloba* leaves on spore germination were similar to mycelium growth. At equal concentrations of 200 µM, the effects of allelochemicals of *Ginkgo biloba* leaves on spore germination were different Myristic acid, 9-octadecenoic acid, eicosanoic acid promoted the spore germination of *Fusarium* wilt. 2-hydroxypropionic acid, benzoic acid, 3-hydroxybenzoic acid, 3,4-dihydroxy-cinnamic acid and n-Hexadecanoic acid inhibited the spore germination of *Fusarium* wilt. With the increase in incubation time, allelopathy of allelochemicals significantly increased on spore germination. For 9-octadecenoic acid and eicosanoic acid, their stimulatory effects on the spore germination of *Fusarium* wilt were significantly higher at 9 and 11 h than at 7h. While the benzoic acid, 3-hydroxybenzoic acid and 3,4-dihydroxycinnamic acid were very inhibitory than above two compounds. However, the 3,4-dihydroxy-cinnamic acid was most inhibitory among all compounds.

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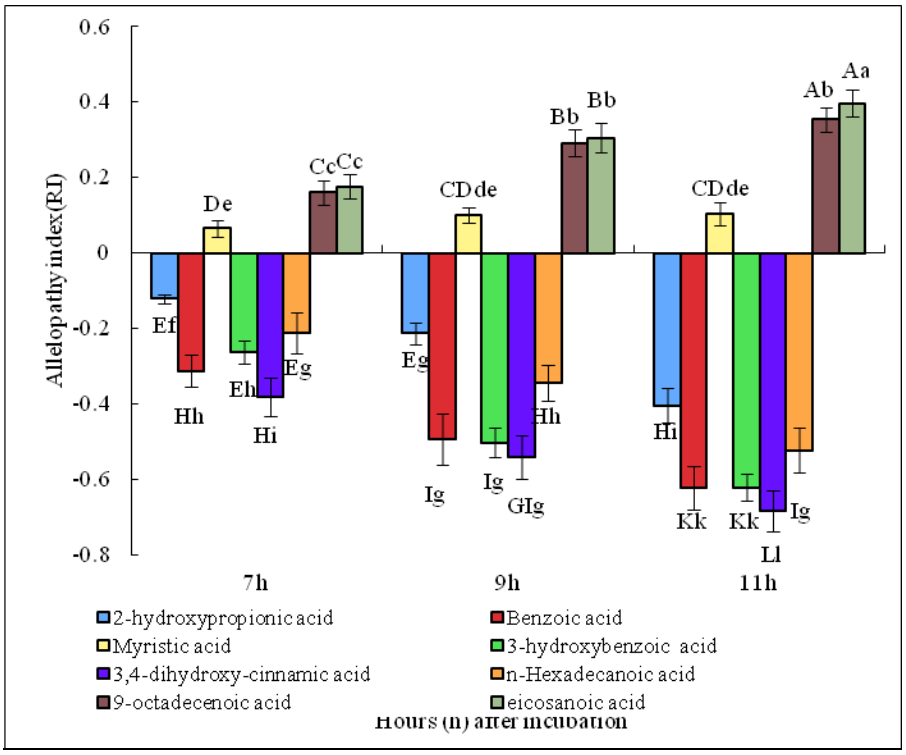

Figure 5. Effects of allelochemicals of gingko leaf extract on spore germination of seedling blight causing fungus

A study found (9,19) that the autotoxic allelopathic potential was greatest in monoculture soil, treated with fertilizer and was least in soil treated with farmyard manure. An analysis showed that fertilizer was conducive to the growth of microbial flora that utilizes amino acids and amines as carbon sources, while farmyard manure was good for the growth of microbial flora that utilize carbohydrates, fatty acids and phenolic acids as

carbon sources (1,36,37). Plants produce numerous low molecular weight compounds, which have the special function of anti-pathogen (7,23). Our results showed that *F. oxysporum* was suppressed by the allelochemicals in *G. biloba* leaves and both the mycelia growth and spore germination were inhibited. The decomposition of gingko leaf biomass in soil, fertilizes the soil and improves the soil structure. Moreover, the decomposing gingko leaves release chemicals that could influence the soil microbial structure and population.

CONCLUSIONS

Ginkgo biloba leaves extract at 0.04 g l^{-1} concentration inhibited the mycelium growth and spore germination of *Fusarium* wilt. In extracts of *G. biloba* leaves, many compounds and 22- allelochemicals [2-hydroxypropionic acid, benzoic acid, myristic acid, 3-hydroxybenzoic acid, 3,4-dihydroxy cinnamic acid, n-hexadecanoic acid, 8-octadecenoic acid, eicosanoic acid] were detected. The application of 2-hydroxypropionic acid, benzoic acid, 3-hydroxybenzoic acid, 3,4-dihydroxy-cinnamic acid and n-Hexadecanoic acid inhibited the mycelium growth and spore germination of *Fusarium* wilt. While the application of myristic acid, 9-octadecenoic acid, eicosanoic acid promoted the mycelium growth and the spore germination of *Fusarium* wilt.

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