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Phytoalexins produced by alfalfa and their role in prevention of spring black stem and leaf spot by *Ascochyta imperfecta*

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Summary

Two phytoalexins were isolated and purified from alfalfa (Vertus) sprayed with of $CuCl_2$. One was identified as medicarpin by ultraviolet (UV)-spectrometry, gas-liquid chromatographic and mass-spectrometry (GC-MS), and high resolution mass-spectrometry. The other was suggested to be sativan (SA) by UV-spectrometry and GC-MS. Six mg of medicarpin and 3 mg of SA were obtained from 5 kg of alfalfa.

Phytoalexin production by alfalfa (three kinds of the cultivar) inoculated with *Ascochyta imperfecta* No.6 was examined for standing change. Medicarpin and SA accumulated earlier and in greater quantity in CUF101 than in Europe or Thor after inoculation with the fungi.

The effects of medicarpin and SA on the growth of 6 strains of the fungi were examined, but were found to be either weak or undetectable. From these results, it may be concluded that medicarpin and SA are produced in alfalfa by inoculation with these fungi, but that they are unable to inhibit invasion by this organism in this plant.

Introduction

Spring black stem and leaf spot disease in alfalfa (*Medicago sativa* L.) caused by *Ascochyta imperfecta* is found throughout Japan and is a particularly serious disease in warm region grass[6),14)].

It is known that several plant species respond to inoculation with various fungi to produce fungitoxic compounds that have been designated as phytoalexins which are postulated to play a primary role in the resistance to disease.

Medicarpin, sativan, vestitol, sativanone and liquiritigenin[2)], and coumesterol[13)] have all been reported as phytoalexins in alfalfa. However the role of these phytoalexins in infection by *A. imperfecta* has not yet been examined.

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In this study, the authors identified two alfalfa phytoalexins produced by spraying with $CuCl_2$, examined for standing change of these phytoalexins after inoculation with the fungi, and tested for their antifungal activity.

Materials and Methods

Alfalfa

Four kinds of alfalfa cultivar (Vertus, CUF101, Thor and Europe) were used in this experiment. Fertilizer [NH_4NO_3 (4.3g), K_2SO_4 (3.7g), $NaH_2PO_4 \cdot 2H_2O$ (8.8g), $CaCO_3$ (7.1g), and $MgCO_3$ (2.5g)] and soils were mixed in Wagner pots covering a space of 1/5,000 a. The alfalfa seeds were sown in the pots and grown at 25℃ in a greenhouse. The Vertus seeds were purchased from Yukijirushi Shubyo CO., Sapporo. The CUF101 was supplied by Dr.S. Inami of the Aichi-ken Agricultural Reserch Center, and the Thor and Europe were supplied by Dr. Y. Takeda of the Hokkaido Prefectural Konsen Agricultural Experiment Station.

Phytoalexins: extraction method from alfalfa

Twenty grams of the epigeal portion (leaf and stem) of alfalfa (Vertus), two months after sowing, were placed in a plastic tray with a wet filter paper lying on the bottom. For the induction of the phytoalexins, the plants were sprayed with 20 ml of 3×10^{-3}M $CuCl_2$ solution containing 0.05% Tween 20. The plants were maintained at 25℃ for 36 hrs in a moist chamber under dark conditions.

The extraction of the phytoalexins from the plants was performed by the method described by Khan and Milton[9]. The extract was dissolved with 4 ml of 60% ethanol, and the solution adjusted to 25% ethanol solution in concentration.

The purification method of the phytoalexins was similar to that described by Edwards and Strange[4]. The ethanol solution was applied to a conditioned Waters Sep-Pak C_{18} cartridge column (Millipore, Milford) washed with 5 ml of methanol and water, washed with 5 ml of 25% ethanol, and eluted with 1 ml of acetonitrile. The eluted solution was concentrated at 300 $\mu\ell$ under N_2 stream.

Chromatographic technique

The solution containing the phytoalexins was subjected to thin-layer chromatography (TLC) on silica gel 60GF_{254} plates (Merck, 500 μm thick). The plates were developed 2 times with *n*-pentane: ethyl ether: acetic acid (75:25:1, v/v)[10] and sprayed with diazotized *p*-nitroaniline reagent[11]. The areas showing their color reactions were scraped from the unsprayed plates, and the phytoalexins in the gel were extracted into 95% (v/v) ethanol. These ethanolic extracts were concentrated after filtration and spotted onto the silica gel plates. The plates were developed 4 times with chloroform: carbon tetrachloride (3:1, v/v)[8] and sprayed with the *p*-nitroaniline reagent. The plates were then sprayed on any unsprayed places with spore suspension of *Cladosporium harbarum*[17], which was cultivated on potato-dextrose-agar (PDA) at 25℃ for two weeks. The spore suspension contained 3 % glucose and 0.3 % yeast extract. These plates were incubated in a moist chamber at 25℃ for 4-5 days. The bands showing the color reactions by the *p*-nitroaniline reagent and antimicrobial activity were scraped from the unsprayed plates, and the phytoalexins extracted into ethanol. The ethanolic solution was concentrated to dryness *in vacuo*, and the residue was dissolved into acetonitrile: water (40:60, v/v).

Purification of phytoalexins by preparative high-performance liquid chromatography (HPLC)

The solution discovered from the TLC plates was subjected to preparative HPLC. Inertsil PREP-ODS (20.0×250 mm, GL Science, Tokyo) was used as a column. An Hitachi L-6000 pump and an Hitachi L-4000 (UV 280 nm) detector were used. A mixed solution of acetonitrile: deionized water (40:60, v/v) was applied as the effluent at a flow rate of 20 ml/min.

Ultraviolet spectrometric analysis

The ultraviolet spectra of the phytoalexins purified by HPLC were taken with an UV-visible recording spectrometer UV-160 (Shimazu Co., Kyoto), using 95 % ethanol solution containing phytoalexins.

Gas-liquid chromatographic and mass spectrometric analyses

After the *o*-trimethylsilylation[8] of the phytoalexins with hexamethyldisilazane: trimethylchlorosilane (1:2, v/v), the trimethylsilylated (TMS) derivatives were injected into a DB-1 column (0.25 mm×30 m; J&W Scientific Co., Folsom). The column oven temperature was programmed from 100 to 300℃ at 5℃/min, and held at 300℃ for 5 min. An Hitachi G-3000 gas chromatograph equipped with a hydrogen-frame ionization detector was used for the analyses. The gas chromatographic and mass spectrometric analyses were performed with an Hitachi M-2500 machine. The mass spectra of the TMS derivatives of the phytoalexins were recorded at an electron energy of 70 eV and an ion source temperature of 150℃. Helium was used as the carrier gas.

High resolution mass spectrometric analysis

A high resolution mass spectrum was taken with an Hitachi M-80B mass spectrometer by direct injection. The electron energy of the ion source was 70 eV. Perfluorokerosene was used as a reference.

Change in amount of alfalfa phytoalexins after spraying with spore suspension of *A. imperfecta*

Three kinds of alfalfa cultivar, CUF101 (group Ⅰ; has low cold resistance and grows in warm regions), Europe (group Ⅱ; has a cold resistance similar to group Ⅰ, but a greater productive capacity), and Thor (group Ⅳ; has high cold resistance and grows in cold regions), with different characteristics[15] regarding habitat, yield and cold resistance, etc., were grown over a period of 6 weeks. About one gram of leaves was collected from each cultivar, respectively.

One ml of spore suspension (2×10^6/ml) of *A. imperfecta* No.6 grown on PDA was sprayed onto the leaves. The suspension solution contained 1% glucose, 0.1 % asparagine, and 0.05 % Tween 20. The control leaves received drops of the suspension solution only. The leaves were incubated at 25℃ under dark conditions in a moist chamber, and the amounts of phytoalexins produced were measured at 0, 6, 12, 24, 36, 48, 72 and 96 hrs after spraying.

The samples for analysis were obtained by performing extraction from the leaves and purification by C_{18} cartridge column in the manner described above. The eluates from the column were evaporated to dryness *in vacuo*, and the residue dissolved in acetonitrile: water (40:60, v/v). This solution was then subjected to HPLC. Hitachi Gel #3056 was packed in a stainless steel column (4×250mm). A mixed solution of acetonitrile: deionized water (40:60, v/v) was used as the effluent at a flow rate of 1 ml/min.

The phytoalexins purified by the preparative HPLC were dissolved in acetonitrile: water (40:60, v/v) at concentrations of 10, 100 and 500 ppm. A calibration curve was made from these analyses by HPLC. All the data procedures were determined with an Hitachi D-2500 chromato integrator.

Tests for antifungal activity of phytoalexins

Inhibition tests on the mycelial growth of 6 strains (No.1-6) of *A. imperfecta* incubated on PDA for 7 days were performed using the method of Higgins[5], and Khan and Milton[10]. The cultures containing PDA and a given amount of medicarpin or SA were incubated in the dark at 25℃ for 7days. Each colony diameter was measured and the results described in terms of percentages compared with that of the controls.

Results

Identification of phytoalexins in alfalfa

An orange color spot (Rf value of approximately 0.58) was observed on the TLC plate when it was developed two times with *n*-pentane: ethyl ether: acetic acid (75:25:1, v/v). When the eluate from the gel was developed three times with chloroform: carbon tetrachloride (3:1, v/v), two spots that had Rf values of approximately 0.21 (orange) and of 0.27 (yellow) were detected on the TLC plate. These spots showed antifungal activity to the spore of *C. harbarum*.

The eluates from the gel corresponding to Rf 0.21 and 0.27 were subjected to preparative HPLC. As shown in Fig. 1, a big peak was found at approximately 17 min in the former, and 31 min in the latter. These eluates were collected and used for the analyses described below.

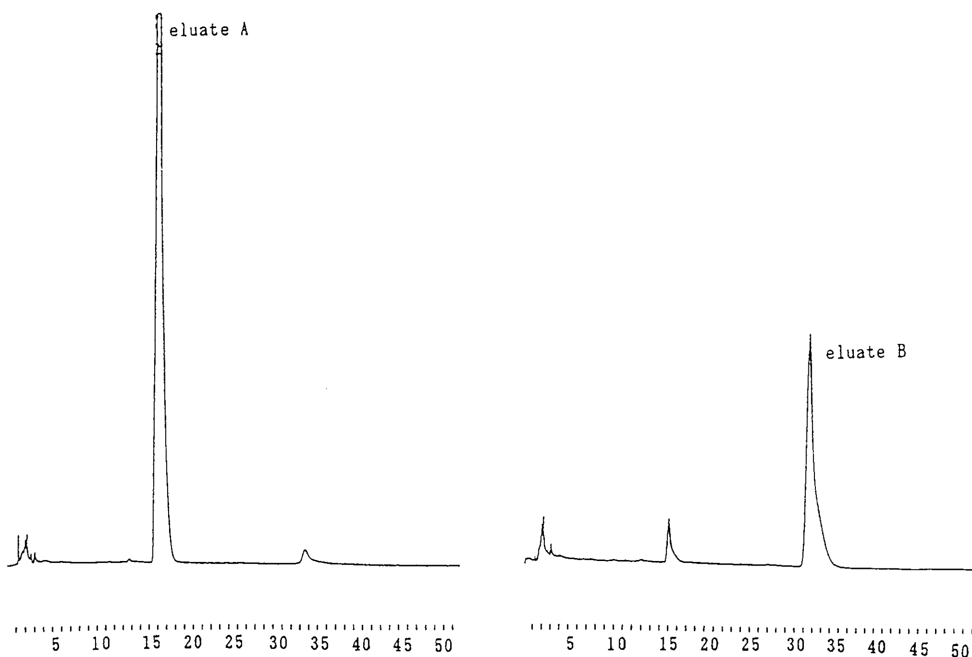


Fig. 1 High performance liquid chromatograms of eluates from silica gel corresponding to Rf 0.21 (left) and 0.27 (right).

The UV spectra for eluate A (the former) and B (the latter) showed absorptions of 211 and 286 nm, and 209 and 283 nm, respectively. They were almost the same as the spectra of medicarpin (213, 282 and 287 nm) and sativan (208, 227, 280 and 284 nm) reported by Bailey and Mansfield[2].

The TMS derivatives of eluates A and B were subjected to GC-MS. The gas chromatograms and mass spectra of these eluates are shown in Fig. 2 and Fig. 3, respectively. In the spectrum of peak 1 (29.1 min) from eluate A in Fig. 1, a molecular ion (M^+ =270) and prominent ions at m/z 255, 226, 207, 161, 148, and 147 were obtained. This was analogous to that seen in free medicarpin, as reported by Ingham[7], and Vaziri *et al* [15]. In peak 2 (30.1 min), a molecular ion (M^+=342) and prominent ions at m/z 327, 219, 206, 161, 148, and 73 were observed, which agreed with that of TMS-medicarpin obtained previously by Edwards and Strange[4]. In the spectrum of peak 2 (30.6 min) from eluate B, a molecular ion (M^+=286) and prominent ions at m/z 164, 151, 149, 121 and 91 were observed. This was essentially identical with that for free sativan, as previously reported by Bonde *et al*[3], and Ingham and Millar[8]. The spectrum of peak 3 (31.5 min) had a molecular ion peak (M^+=358) and prominent ions at m/z 207, 206, 164, 151, 149, 121, 91, and 73. Therefore, it was presumed to be the spectrum of TMS-sativan from its cleavage pattern.

In the high resolution mass spectrometric analysis of eluate A, the analytical value of the molecular weight was 270.0892, and the difference was -0.002 units compared with the theoretical value (270.0912) in the molecular formula ($C_{16}H_{14}O_4$) of medicarpin.

That from eluate A was identified as medicarpin by UV-spectrometry, GC-MS and high resolution mass spectrometry. The other, from eluate B, was suggested to be sativan (designated as SA) by UV-spectrometry and GC-MS. Six mg of medicarpin and 3 mg of SA

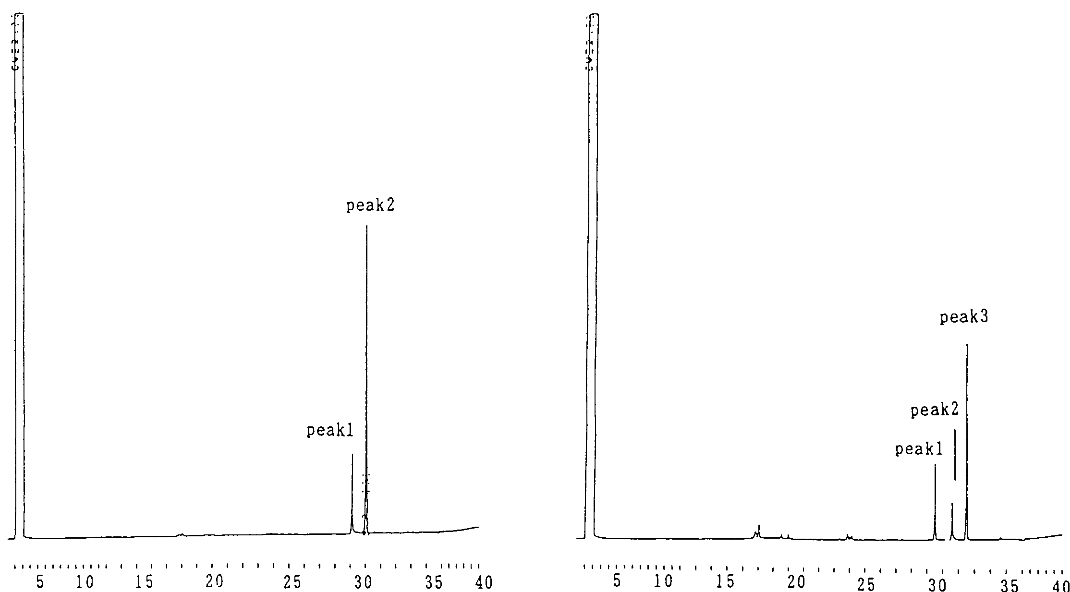
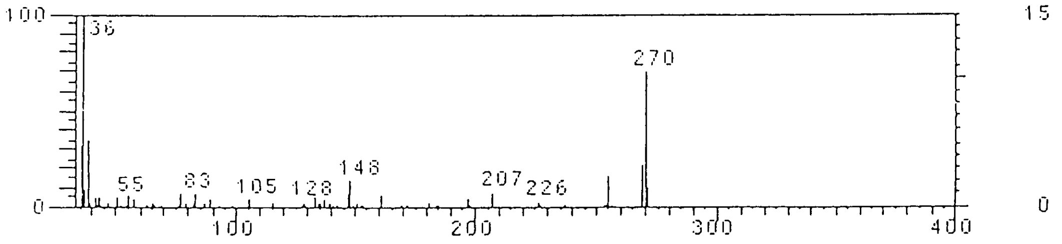
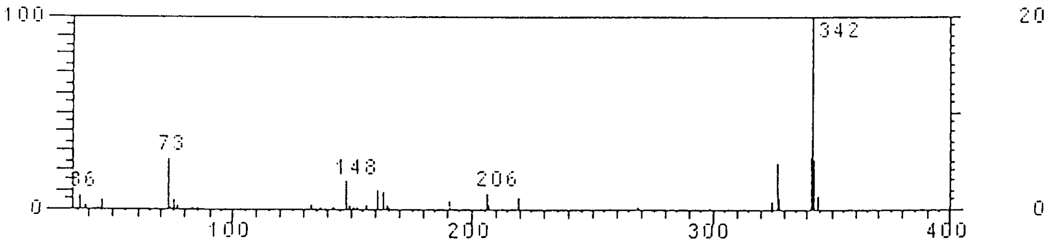


Fig. 2 Gas liquid chromatograms of eluate A (left) and eluate B (right).

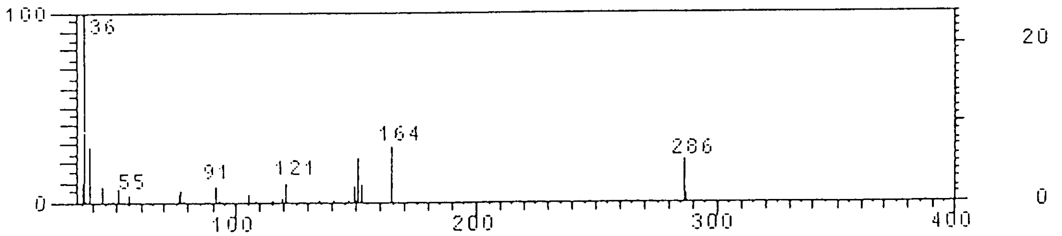
Peak 1 in eluate A



Peak 2 in eluate A



Peak 2 in eluate B



Peak 3 in eluate B

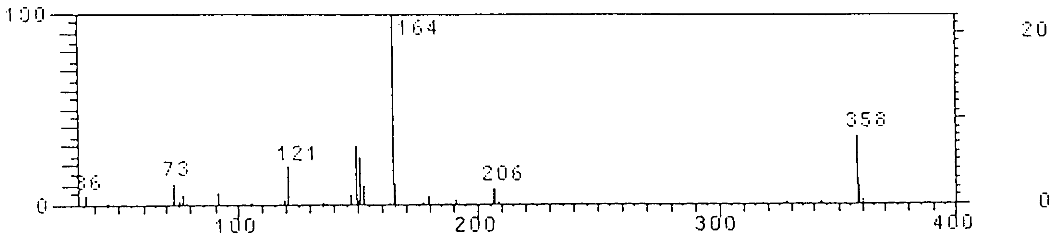


Fig. 3 Mass-spectra of peaks in Fig.2.

were obtained from 5 kg of alfalfa (cv.Vertus) by spraying with $CuCl_2$.

Changes in the amount of phytoalexins after spraying with spore suspension of *A. imperfecta*

We investigated change with the passage of time in phytoalexin production from the alfalfa cultivars after spraying with spore suspension of *A. imperfecta* No.6. The high-performance liquid chromatograms of the extract from two cultivars of alfalfa (Thor and CUF101) at 72 hrs after spraying are shown in Fig. 4. The maximum production of medicarpin

was obtained at 12 hrs in CUF101 (10.9 $\mu g/g$), at 96 hrs in Europe (6.1 $\mu g/g$) and at 72 hrs in Thor (5.0 $\mu g/g$) after spraying (Fig. 5). The maximum production of SA was obtained at 36 hrs in CUF101 (20.6 $\mu g/g$), at 72 hrs in Europe (6.5 $\mu g/g$), and at 72 hrs in Thor (7.6 $\mu g/g$) after spraying (Fig. 5).

Antifungal activity of phytoalexins

The effects of medicarpin or SA on the mycelial growth of *A. imperfecta* were tested at concentrations of 0 (controls), 25, 50 and 100 ppm (Table 1). The degrees of growth of the *A. imperfecta* were expressed as a percentage of the controls. The degrees of the 6 strains (No. 1-6) of *A. imperfecta* were 67-101 % for 100 ppm of medicarpin, and 69-107% for 100 ppm of SA.

Discussion

Phytoalexins are produced in alfalfa by ultraviolet irradiation or treatment with heavy metals [9),12)], in addition to inoculation with fungi. It has been reported that the adjustment of metals as an inducer is simple, and that phytoalexins such as medicarpin and sativan are produced in rich quantities by such treatment[9),12)]. Six mg of medicarpin and 3 mg of SA were obtained from 5 kg of alfalfa (Vertus) by spraying with $CuCl_2$ in this experiment.

Medicarpin and SA purified by preparative HPLC were detected as two or three peaks by gas chromatography (GLC) (Fig. 2), after they had been converted to their TMS derivatives. From the spectra of the GC-MS of these peaks, free medicarpin and SA and their TMS derivatives were recognized to coexist. It is presumed that the trimetylation of these substances is insufficient. By MS-analysis, peak 1 (29.5 min), which was preceded by TMS-SA on the GLC, appeared to be carboxylic acid ester (M.W. 390), a contamination from plastic vials

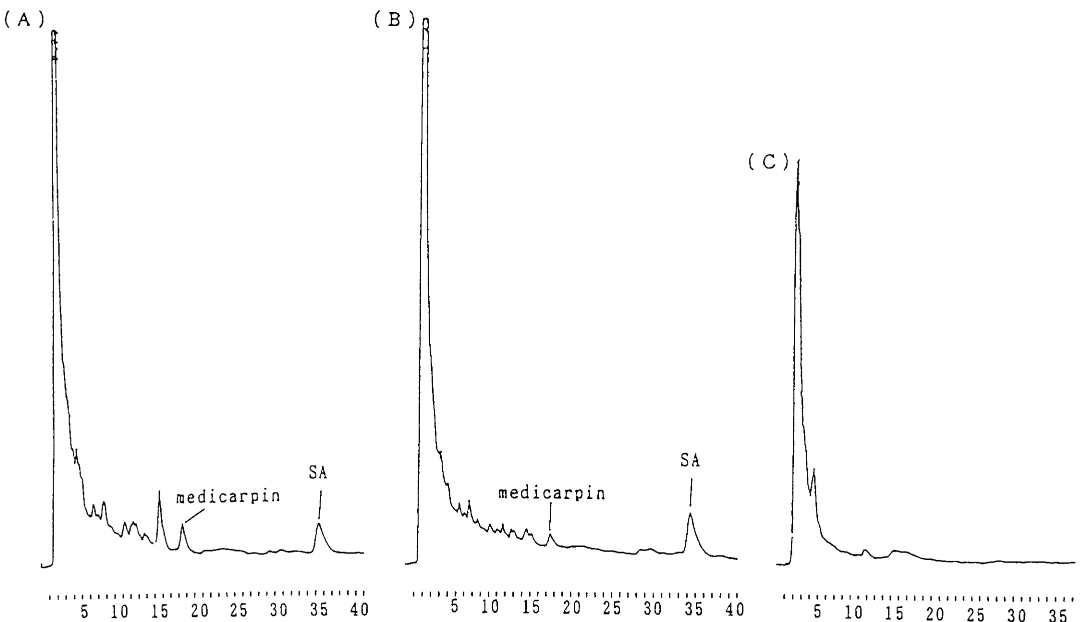


Fig. 4 High performance liquid chromatograms of extracts from Thor (A), CUF101 (B) at 72 hrs after inoculation with spore suspension of *Ascochyta imperfecta* No.6, and extract from Thor (C) at 72 hrs after inoculation with water solution.

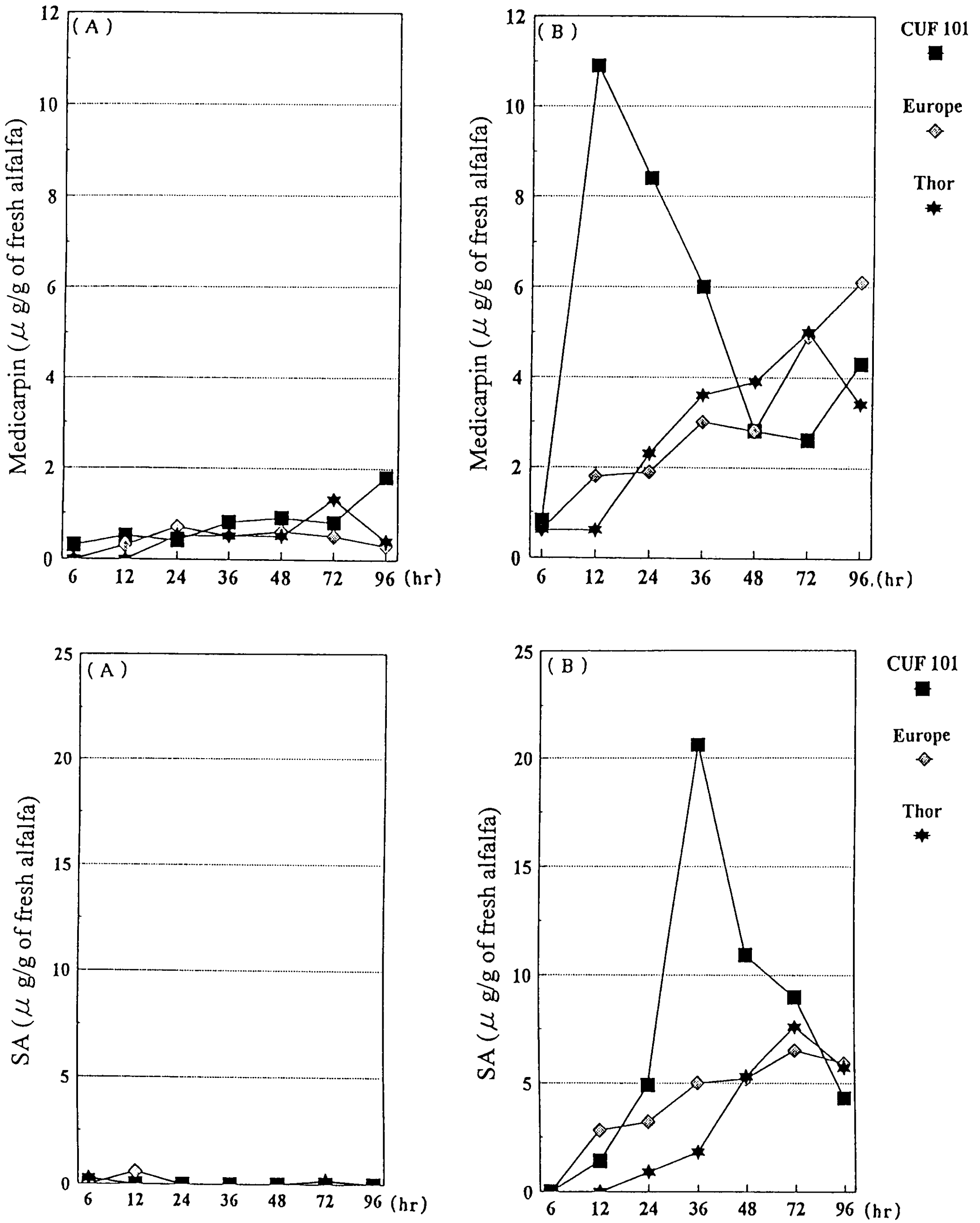


Fig. 5 Change in medicarpin and SA production in alfalfa cultivars (CFU101, Europe and Thor) with time after inoculation with (A) water solution and (B) spore suspension of *Ascochyta imperfecta* No.6.

found in any extraction process.

Host plant cells exhibit hypersensitive reaction, and phytoalexins are produced immediately from the adjacent cells of hypersensitive death cells by the invasion of fungal mycelia.

Table 1 Effect of medicarpin and SA on mycelial growth of each isolate of *Ascochyta imperfecta.*

Isolate No.	Medicarpin				SA			
	0	25	50	100(pm)	0	25	50	100(ppm)
No.1	100	86	81	71	100	86	81	81
No.2	100	89	89	67	100	106	83	72
No.3	100	100	100	101	100	113	100	107
No.4	100	100	100	94	100	101	101	101
No.5	100	91	82	82	100	86	77	73
No.6	100	118	101	75	100	113	106	69

Values are given as % of controls.

The phytoalexins produced are accumulated in plant cells in enough amounts to inhibit the extension of the invasive mycelia, and play an important role in the first stage of infection in plants[1]. In the combination of alfalfa and *Phytophthora megasperm* f. sp. *medicaginis*[16], phytoalexins were produced more rapidly and more abundantly in resistant races of alfalfa than in sensitive ones. In our study, differences in the amount and speed of phytoalexin production among three varieties (CFU101, Europe, and Thor) were tested by spraying with spores of *A. imperfecta* No.6. Medicarpin and SA were accumulated earlier and in greater quantity in CUF101 than in Europe or Thor.

Phytoalexins are antifungal substances. It has been reported that medicarpin and sativan inhibit the growth of *Helminthosporium turcicum*[8], and that the ED_{50} of medicarpin and sativan is 25 ppm and 15 ppm, respectively. It has also been reported that these two substances inhibit the growth of *Verticillium albo-atrum*[9] and *Verticillium dahliae*[9,10]. However, medicarpin[5] could only slightly (0-12 %) inhibit the growth of *Phoma herbarum* var. *medicaginis, Stemphylium botryosum, Colletotrichum trifolii*, and *Leptosphaerulina briosiana* by levels of 75 μg/ml of media. The effects of medicarpin and SA on the growth of 6 strains of *A. imperfecta* were examined in the present study. They hardly showed any effect on the growth of the fungi at a level of 100 ppm. When the three cultivars of alfalfa were inoculated with the six strains of *A. imperfecta*, all the cultivars were infected and lesions were formed, although a small difference in the number of lesions on the leaves was observed (no data is shown in this text).

From the data described above, it may be concluded that medicarpin and SA are produced in alfalfa by inoculation with *A. imperfecta*, but that they are unable to inhibit the invasion of *A. imperfecta* in the plant. It has been reported that *A. imperfecta* has a mechanism for decomposing medicarpin[5]. However, whether the strains used in this study decomposed such substances remains open to question. This mechanism deserves further study.

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要　　約

塩化第二銅を噴霧されたアルファルファ（Vertus）から二種類のファイトアレキシンが抽出・精製された。一つは紫外線吸収スペクトル，ガスクロマトグラフィー－マススペクトロメトリー（GC-MS），高分解質量スペクトルから medicarpin と同定された。もう一つは紫外線吸収スペクトル，GC-MS から sativan (SA) と推定された。5 kg のアルファルファから 6 mg の medicarpin，3 mg の SA が得られた。アルファルファの茎枯病の原因菌である *Ascochyta imperfecta* No. 6 株をアルファルファの 3 品種に接種し，ファイトアレキシンの産生量を経時的に調べた。Europe，Thor よ

りCFU 101において，medicarpin及びSAはより速くより多く産生された。*A. imperfecta* 6菌株の発育に対するmedicarpin及びSAの影響について調べた。これらの物質は100 ppmの濃度においても本菌の発育をほとんど抑制しなかった。以上の結果から，*A. imperfecta*の感染によりアルファルファからmedicarpin及びSAは産生されるが，これらの物質は，本菌のアルファルファへの侵入をほとんど抑制しないと思われた。