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Isolation and phytotoxic effects of helvolic acid from plant pathogenic fungus *Sarocladium oryzae*

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Abstract. In this study, we isolate helvolic acid, an antibiotic metabolite, from the rice sheath rot pathogen *Sarocladium oryzae*. The fungus is cultured in potato-sucrose broth. The metabolite is extracted by ethyl acetate, and its antibiotic activity is tested with *Bacillus subtilis*. Helvolic acid is soluble in chloroform, acetone, ethyl acetate, methanol, ethanol, and alkali water. The substance is stable at an active temperature of 22°C–100°C and is also active at various hydrogen ion levels (pH 3–11). Also, helvolic acid is identified with thin-layer chromatography and further characterized by UV, IR, NMR, and Mass spectroscopy. The toxic effects of helvolic acid on seedlings of Graminae include growth retardation and chlorosis. These effects are reduced about 40% by treating with Hoagland's solution and can be reduced to around 30% by treating with magnesium solution.

Keywords: Antibiotic; Helvolic acid; Phytotoxic substance; Plant pathogenic fungus; *Sarocladium oryzae*.

Introduction

Sarocladium oryzae is a cause of sheath rot in rice (Gams and Hawksworth, 1975; Rahman et al., 1982). The serious infection of this fungus on rice plants can result in the sterility of rice, as in Taiwan, where the crop suffered a 20–60% loss (Tschen and Wen, 1980). Our present study indicates that a symptom similar to the sterility of rice is induced by infiltrating the culture filtrate of *S. oryzae* (Tschen and Wen, 1980).

Helvolic acid (fumigacin), an antibiotic, is produced by some fungi including *Aspergillus fumigatus, Cephalosporium caeruleus*, and *Emericellopsis terricola* (Chain et al., 1943; Cole and Cox, 1981); however, the toxic property of the substance to plants is unknown. Recently we found that *S. oryzae* also produced helvolic acid (Figure 1). In this study, we prepare helvolic acid from *S. oryzae* and test its inhibitory effects on the growth of several species of seedlings.

Materials and Methods

Chemicals and Seeds

The chemicals of growth media for microorganisms were purchased from Difco, USA. The analysis chemicals were purchased from E. Merck, Germany and Sigma, USA. The seeds for bioassay were provided by the Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Taichung, Taiwan.

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Microorganisms and Growth Media

Sarocladium (*Acrocylindrium*) *oryzae* (Saw.) W. Gams & D. Hawksw. (Gams and Hawksworth, 1975) isolate AO-3 was grown on yeast extract agar (YA: 4 g yeast extract, 10 g malt extract, 4 g glucose, 20 g agar/l, pH 7.3). *Bacillus subtilis* ATCC 6051 was grown on nutrient agar (NA:

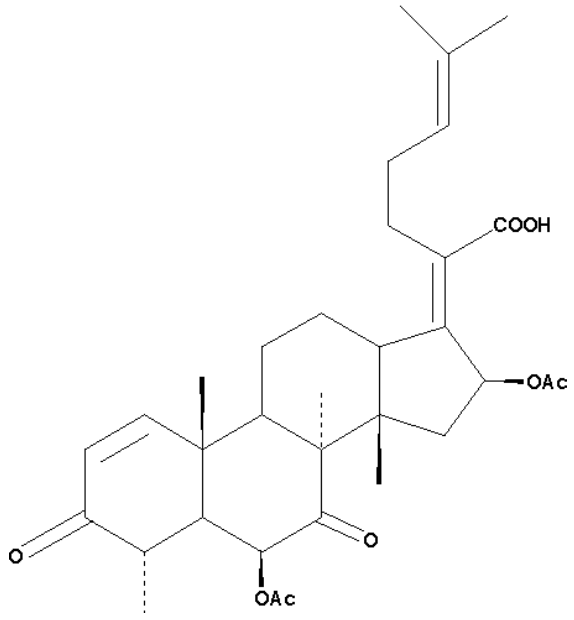

Figure 1. The chemical structure of helvolic acid from *Sarocladium oryzae*.

3 g beef extract, 5 g peptone, 2.5 g NaCl, 20 g agar/l, pH 7.3–7.5). The cultures were set at 27°C.

Crude Extract

Sarocladium oryzae was grown on YA plate for a week. The fungal culture was punched out with a 75 mm cork borer, and ten pieces of the culture disk were placed into a 500 ml Erlenmeyer flask containing 100 ml of potato-sucrose-peptone broth (PSP: potato 200 g, sucrose 15 g, peptone 5 g, Cu $(NO_3)_2$•$3H_2O$ 0.1 g, Na_2HPO_4•$12H_2O$ 20 g, distilled water 1l) (Tschen and Wen, 1980). This broth was shaken with an orbital shaker (Lab-Line, USA) at 240 rpm and 27°C for 4 days. The broth culture was filtrated with glass wool to remove the mycelia. The culture filtrate was extracted three times with ethyl acetate. The resulting extract was then concentrated as crude helvolic acid using a rotary evaporator (Büchi, Switzerland) at 45°C. The large scale production of helvolic acid was conducted with a 7.5, l-table fermentor (New Brunswick, USA, Model MF107). The vessel of the fermentor contained 4 l PSP broth. The fermentation was conducted at 27°C. Stirring continued at 200 rpm, and 4 liters per min of aeration was performed.

Purification of Helvolic Acid

Crude helvolic acid was dissolved in ethyl acetate and loaded on a silica gel 60 column (2.5 × 50 cm, 230–400 mesh, ASTM, Merck). It was eluted with 200 ml ethyl acetate at a flow rate of 1 ml/min. The antibiotic fractions against *B. subtilis* were collected by an automatic fraction collector (model 2112, LKB). The antibiotic substance was concentrated from those fractions with the rotary evaporator at 45°C. This antibiotic substance was dissolved in ethyl alcohol at 45°C, and then the antibiotic solution was collected with the filter paper (Whatman No. 1). The ethanol fraction was put into the refrigerator over night, and, finally, white crystals of helvolic acid were collected by filtration.

Chemical Analysis

Thin-layer chromatography of helvolic acid followed the method of Stahl (1969) using TLC aluminium sheets (silica gel 60 F254, layer thickness 0.2 mm, Merck) and a developing solvent system containing a mixture of chloroform/glacial acetic acid, 95/5 (v/v).

Crystal helvolic acid was dissolved in methanol (mg/ml) whose spectra were analyzed with a UV-visible double beam spectrophotometer (model 200–20, Hitachi, Japan). For measuring infra red spectra, 1 mg helvolic acid was mixed with 200 mg KBr. The mixture was then pressed to become a disk at 15,000 lbs/in^2. Finally, spectra of the substance were measured by a IR-spectral photometer (model 457A, Perkin-Elmer, USA).

NMR spectra were recorded in $CDCl_3$ solution on a Bruker WP-100 spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured at 100 and 25 MHz, respectively. All of the chemical shifts were recorded with respect to internal TMS. Mass spectra were obtained using a JOEL JMS-HX 110 mass spectrometer equipped for fast atom bombardment analysis.

Agar-Diffusion Test

The agar-diffusion tests for antibiotic activities were performed with petri dishes 90 mm in diameter, with 17.5 ml NA medium for the basic layer and 5 ml NA medium for the top-layer (added after solidification of the basic layer) containing *B. subtilis* (Loeffler et al., 1986). Paper disks 6 mm in diameter loaded with 10 µl helvolic acid (175 mg/l) were put on the top-layer overnight. The antibiotic activity expressed the size of inhibition zones.

Bioassay

Seeds were disinfected in a solution of 2% sodium hypochlorite for 15 min. Seeds were then rinsed with distilled water three times. The germination test was conducted in a 9 cm diameter petri dish (Association of Official Seed Analysts, 1981). Thirty seeds were uniformly spread on the filter paper (Whatman No. 1) in which 100 to 1,000 ppm helvolic acid was contained. A randomized design was used with three replications. Petri dishes were set in the growth chamber at 27°C. The light and dark periods were controlled for 12 h each. The growth of seedlings was observed two weeks after planting. The rate of chlorosis seedling was estimated by the method of Loeffler et al. (1986). Hoagland's nutrient solution and its defective solution, containing single element, were used to test reduction of chlorosis on the seedlings.

Results

Isolation of Helvolic Acid

Sarocladium oryzae was continuously cultivated by shaking for two weeks to observe the production of helvolic acid. During the cultivation period, the sample of culture filtrate was accumulated daily to test the antibiotic activity. The antibiotic activity of the culture filtrate was observed from two days after inoculation and rose to a maximum three days after inoculation. Thereafter, the

Table 1. Solubility of helvolic acid from *Sarocladium oryzae* in various organic solvents.

Solvent	Solubility[a]
Acetone	+++
n-Butanol	++
Chloroform	+++
Ethyl acetate	++
Ethanol	++[b]
Methanol	++[b]
Petroleum ether	+
Distiled water, pH 7	+
Distiled water, pH 9	++
Distiled water, pH 11	+++

[a]Solubility tested with mg/ml of helvolic acid. +, slightly dissolved; ++, dissolved; +++, easily dissolved.
[b]Dissolved at 45°C.

activities decreased. The pH-value of the culture filtrate increased daily after inoculation day.

The crude helvolic acid was easily extracted with ethyl acetate from culture filtrates and separated by a silica gel 60 column and eluted with a volume of 200 ml ethyl acetate. A volume of 5 ml fraction under a flow rate of 1 ml/min was collected. Helvolic acid was found in fraction numbers 15–35. Helvolic acid could be purified by crystalization with ethyl alcohol. This substance is white crystalline, m.p. 215°C. Finally, the minimum inhibition concentration to *B. subtilis* was tested at 10 ppm.

Properties of Helvolic Acid

Helvolic acid is soluble in organic solvents including acetone, chloroform, n-butanol, ethyl acetate, ethanol, methanol, petroleum ether, and neutral or alkali water. Table 1 presents the solubility of helvolic acid.

Helvolic acid has stable antibiotic activity. This activity showed no remarkable change when the substance was treated at 100°C or exposed under light for three months (Table 2). The antibiotic activity was totally lost by autoclaving at 121°C under 1.5 kg/cm^2 of pressure for 10 min. Moreover, the antibiotic activity of helvolic acid becomes stable at pH 3 to 11 (Table 3).

Rf-value of crystalline helvolic acid is 0.55 when the thin-layer chromatogram was developed with a solvent system of chloroform/glacial acetic acid (95/5, v/v).

Spectroscopic Characterizations of Helvolic Acid

Helvolic acid produced from *S. oryzae* was identified primarily by comparison with the spectroscopic characteristics reported in the literature (Cole and Cox, 1981).

The largest detectabale ion in the mass spectrum was *m/e* 508 due to M^+-60. UV spectrum taken in methanol displayed two peaks with λ_{max} at 205 nm and 230 nm (Figure 2). IR (KBr) ν_{max} 3550, 3450, 2800–3100 (C-H and C=C-H), 1640–1750 (C=O, OAc), 1430–1460, 1370, 1200–1230 and 1030 cm^{-1} (Figure 3), ^{1}H NMR ($CDCl_3$) δ 0.92, 1.17, 1.26, 1.30, 1.46, 1.67, 1.70, 1.93, 2.10, 5.10, 5.20, 5.80, 5.93, 7.33 ppm (Figure 4A). ^{13}C NMR ($CDCl_3$) CH_3 δ 13.1, 17.8, 18.0, 18.3, 20.5, 20.7, 25.7, 27.5 ppm; CH_2 δ 23.9, 25.9, 28.3, 28.6, 40.7; CH δ 40.4, 41.8, 47.2, 49.4, 73.5, 73.8, 122.8, 127.8, 157.4; quaternary C δ 38.2, 46.6, 52.7, 130.6, 132.8, 147.4, 169.0, 170.4, 174.3, 201.5 (C=O) (Figure 4B), 208.8 (C=O). The ^{13}C NMR spectrum was partially assigned based on the DEPT experi-

Table 3. Effects of pH value on antibiotic activitiy of helvolic acid from *Sarocladium oryzae*.

pH	Antibiotic activity[a]
3.00	13.2
5.00	11.7
7.00	11.6
8.25	11.9
9.00	11.8
11.00	12.1

[a]mm of diameter of inhibition zone of *Bacillus subtilis* ATCC 6051 tested with 17.5 ng helvolic acid, means from four replicates.

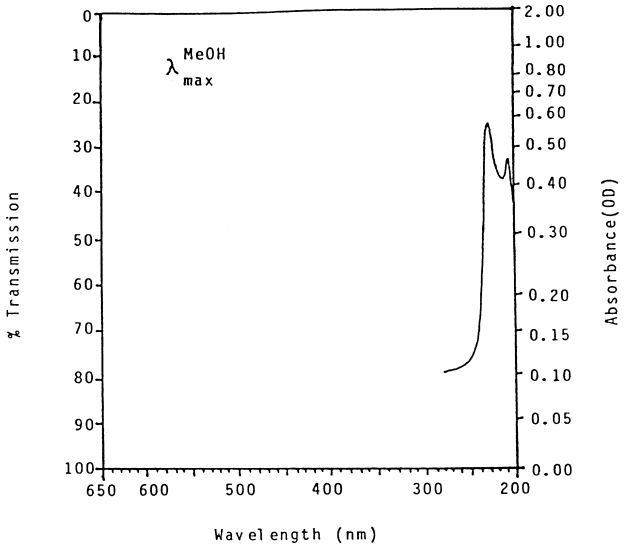

Figure 2. The UV absorption spectrum of helvolic acid from *Sarocladium oryzae* in methanol.

Table 2. Effects of temperature on antibiotic activities of helvolic acid from *Sarocladium oryzae.*

Temperature (°C)	Time of treatment							
	Hour				Day			
	0.5	1	3	5	1	7	30	120
100	13[a]	12	12	12	–[b]	–	–	–
60	13	12	12	12	–	–	–	–
45	13	13	13	13	–	–	–	–
37	–	–	–	–	12	12	12	12
27	–	–	–	–	12	12	12	12
4	–	–	–	–	12	12	12	12
-22	–	–	–	–	12	12	12	12
Dark at 30	–	–	–	–	12	12	12	12
Light at 30	–	–	–	–	12	12	12	12

[a]mm of diameter of inhibition zone of *Bacillus subtilis* ATCC 6051 tested with 17.5 ng helvolic acid, means from four replicates.
[b]Not tested.

ment, and the result is consistent with the reported structure of helvolic acid.

Effects of Helvolic Acid on Growth of Seedlings

The phytotoxic effects of helvolic acid were examined in different species of seedlings, including monocotyledones, dicotyledones, crops, and weeds. The seedlings were treated with 100, 200, 500, and 1000 ppm of helvolic acid to observe the phytotoxic response. Toxic effects were estimated on the basis of reduction of plant height, root length, and root number, or increasing chlorosis rate of seedlings. Chlorosis is a typical symptom brought on by helvolic acid; however, the most prevalent toxic effect to seedlings appears to be the reduction of root

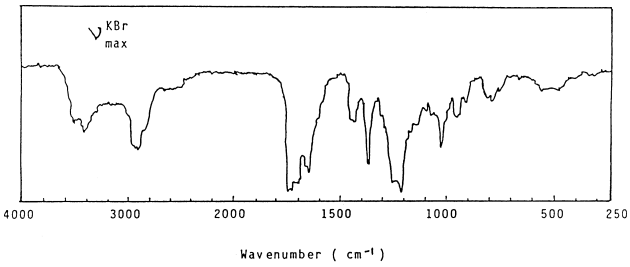

Figure 3. The IR absorption spectrum of helvolic acid from *Sarocladium oryzae* in Kbr.

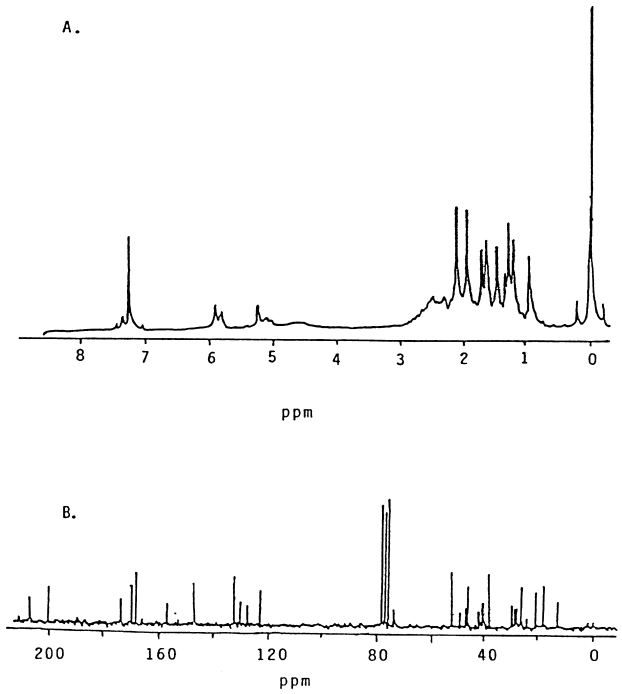

Figure 4. The NMR absorption spectra of helvolic acid from *Sarocladium oryzae* in $CDCl_3$ solution. A, the 100 Mhz ^1H NMR spectrum; B, the 25 Mhz ^{13}C NMR spectrum.

Table 4. Effects of helvolic acid on growth of various species of seedlings.

Plant	Conc. (ppm)	Stem (mm)	Root (mm)	Root No.	Chlorosis (%)
Barnyard grass (*Echinochloa crus–galli*)	0	33.94[a,*]	45.32[a]	3.01[a]	0.00[a]
	200	27.92[b]	25.47[b]	1.64[b]	84.71[b]
	1000	25.76[b]	18.75[c]	1.87[b]	100.00[c]
Goose grass (*Eleusine indica*)	0	8.24[a]	29.74[a]	1.55[a]	0.00[a]
	200	5.81[b]	17.53[b]	1.84[b]	76.62[b]
	1000	4.75[b]	13.52[b]	1.70[ab]	100.00[c]
Rice (*Oryza sativa*)	0	42.42[a]	56.07[a]	7.82[a]	0.00[a]
	200	49.30[b]	60.52[ab]	8.34[b]	35.96[b]
	1000	58.24[c]	67.19[b]	8.49[b]	100.00[c]
Snapweed (*Impatiens walleriana*)	0	9.02[a]	19.81[a]	3.12[a]	0.00[a]
	200	7.11[b]	11.11[ab]	3.11[a]	33.55[b]
	1000	6.67[c]	9.84[b]	2.54[b]	83.33[b]
Tomato (*Lycopersicon esculentum*)	0	23.55[a]	63.09[a]	2.73[a]	0.00
	200	21.57[b]	73.33[b]	1.14[b]	0.00
	1000	20.07[c]	56.01[c]	1.64[ab]	0.00
Bur–marigold (*Bidens bipinnata*)	0	29.76[a]	57.28[a]	1.21[a]	0.00
	200	24.86[b]	50.79[a]	1.50[b]	0.00
	1000	25.88[c]	32.41[b]	1.08[a]	0.00
Pink (*Dianthus chinensis*)	0	4.65[a]	26.43[a]	1.00[a]	0.00[a]
	200	5.00[b]	15.98[b]	1.00[a]	24.81[b]
	1000	5.10[b]	15.76[b]	1.00[a]	61.85[c]

*Values within the column not followed by the same letter are significantly different at 5% level according to Duncan's multiple range test.

Table 5. Effects of nutrient elements and helvolic acid on growth of *Echinochloa crus–galli*.

Nutrient element	Helvolic acid (ppm)	Plant height (mm)	Root length (mm)	Root number	Chlorosis seedling (%)
H_2O	0	33.94^{cd,*}	45.32^{abc}	3.01^{ab}	0.00
	200	27.97^{b}	25.47^{ab}	2.22^{ab}	92.78^{a}
Full**	200	44.74^{a}	21.63^{ab}	2.26^{ab}	51.85^{b}
N***	200	32.33^{ab}	18.78^{b}	1.59^{c}	95.56^{a}
P	200	31.91^{ab}	28.03^{ab}	2.68^{ab}	84.92^{ab}
K	200	37.07^{ab}	31.93^{ab}	2.18^{ab}	90.92^{a}
Mg	200	33.17^{ab}	32.60^{a}	2.71^{a}	60.58^{b}
Fe	200	31.20^{b}	25.47^{ab}	2.50^{ab}	81.35^{ab}
Ca	200	31.08^{b}	19.90^{ab}	2.11^{b}	96.53^{a}

*Values within the column not followed by the same letter are significantly different at 5% level according to Duncan's multiple range test.

**Hoagland's nutrient solution.

***Nutrient solution prepared from Hoagland's solution which contains only a single element (nitrogen source).

length. The phytotoxic effect on seedlings became more and more serious after treatment with an increasing concentration of helvolic acid. The plant species of Gramineae, barnyard grass (*Echinochloa crus-galli*), goose grass (*Eleusine indica*), and rice (*Oryzae sativa*) are all sensitive to helvolic acid. These Gramineae seedlings exhibited the typical chlorosis symptom when they were treated with 100 ppm of helvolic acid (Table 4). The nutrient elements were used to prevent chlorosis formation of barnyard grass. Those grasses were grown either in Hoagland's solution or in the Hoagland's defective solutions containing the single element N, P, K, Mg, Fe, or Ca, in addition to 200 ppm of helvolic acid. The Hoagland's solution and the Hoagland's defective solutions containing magnesium ions were able to effectively reduce chlorosis formation in barnyard grass seedlings. The chlorosis rate dropped about 40% when the seedlings were treated with Hoagland's solution; when the seedlings were treated with magnesium element, the rate fell 30% (Table 5). In this case, magnesium is an important element to prevent helvolic acid from being affected.

Chloroplasts in the paraffin sections prepared from chlorosis seedlings were histologically observed. The formation of chloroplast in the chlorosis tissues was subsequently inhibited. The chlorosis tissues of barnyard grass were lacking in chloroplast when the grass seedling was grown in 200 ppm of helvolic acid.

Discussion

Zapeck's solution was reported as a favorable culture broth to growth of *S. oryzae* (Mohan and Subramanian, 1979). However, our results indicated that the medium is inappropriate for producing helvolic aicd. There was no antibiotic activity to be tested in the cultural filtrate after twelve days of cultivation with *S. oryzae*.

Helvolic acid is sensitive to *Bacillus subtilis* and is also stable to heat and stable at various pH levels. The substance is antibiotic active in the cultural filtrate of *S. oryzae*, and during preparation can be detected with an agar-diffusion test. The spectroscopic data of UV, IR, MNR, and MS of helvolic acid from *S. oryzae* are similar to those of helvolic acid produced from *Aspergillus fumigatus, Cephalosporium caerulens,* and *Emericellopsis terricola* (Cole and Cox, 1981).

It is interesting to find that the ^{1}H and ^{13}C NMR spectra of helvolic acid have never been fully analyzed in the literature. The ^{13}C NMR spectrum was successfully assigned to the carbon category based on DEPT experiments in this field. Further assignment by modern NMR technology is promising.

Our results indicated that helvolic acid induces seedlings of rice, grasses, and weeds to become chlorotic. Some seedlings are hosts of *S. oryzae* including *Echiochloa colona, Eleusine indica, Monochloria vaginalis, Cyperus iria* (Balakrishnan and Nair, 1981; Rahman et al., 1982). Similar results were observed with helvolic acid from *A. fumigatus* affected maize (Berestetskii, 1974). Magnesium is an important factor preventing seedlings from becoming chlorotic. Helvolic acid apparently attacks the formation of chloroplasts. Magnesium ion is a necessary component of chlorophyll, which reacts with protoprohyrin IX in the pathway of chloroplast biosynthesis (Castelfranco and Beale, 1981). Magnesium ion may also activate the enzymes of chlorophyll biosynthesis and function as an antagonist against the toxic effects of helvolic acid on seedlings.

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水稻葉鞘腐敗病菌之煙麴黴酸的分離和植物毒性

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吾人從水稻葉鞘腐敗病菌分離出一種抗生物質，煙麴黴酸。真菌以馬鈴薯蔗糖培養液培養生產煙麴黴酸。以乙酸乙酯自培養基中萃取煙麴黴酸，並利用枯草桿菌測試抗生活性。煙麴黴酸可溶於氯仿、丙酮、乙酸乙酯、甲醇、乙醇和鹼性水，它在 22–100℃ 和 pH 3–11 兼性仍然保持穩定。煙麴黴酸是依據紫外線光譜儀、紅外線光譜儀、核磁共振儀和質譜儀顯之吸收光譜而鑑定。煙麴黴酸的毒性在禾本科植物幼苗呈現萎化和黃化。這種毒性現象經霍克蘭溶液和鎂離子溶液處理後，分別減輕症狀百分之四十和百分之三十。

關鍵詞：煙麴黴酸；水稻葉鞘腐敗病菌；植物毒物；抗生素；植物病原真菌。