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Biological activities of the diethyl ether soluble toxin produced by *Helminthosporium sativum*

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*Helminthosporium sativum*이 생성하는 D-toxin의 생물학적 활성

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ABSTRACT: Diethyl ether soluble toxin produced by *H. sativum* had the characteristics of helminthosporal as based on UV, GC-MS, and chemical analysis, but was not a helminthosporal. It was speculated that it was a polymer of helminthosporal. It stimulated the productions of reducing sugar in the barly endosperm like gibberellic acid, but acted in the responses on the barley roots and coleoptiles like gibberellic acid. It seemed to be involved in Foliar and Root rot diseases with host specificity, based on the analysis of linear regression.

KEYWORDS: *Helminthosporium sativum*, Helminthosporal, Diethyly soluble toxin, Phytotoxin.

The concept of (phyto-) toxins produced by *Helminthosproium sativum* P. K. & B. (*Cochliobolus sativus*) was first proposed by Ludwig *et al.* (1956) and Ludwig (1957) in Canada. DeMayo *et al.* (1961 ; 1963) extracted and chemically characterized a toxic substance through serial chemical reduction and oxidation reactions and named it 'helminthosporal'. Based on the empirical formula ($C_{15}H_{22}O_2$) and infrared spectra, he concluded that helminthosporal had two aldehyde groups and a double bond. Later, DeMayo *et al.* (1965) reported that the compound toxic to plants was prehelminthosporal, which was converted to helminthosporal during purification procedures. However, helminthosporal was also reported to be directly obtained from the fresh culture filtrates and shown to be toxic to plant tissues (Sommereyns and Closset, 1978). The helminthosporal reduced or inhibited barley germination and growth in young seedlings (Ludwig *et al.*, 1956; Spencer, 1965). Helminthosporal were reported to be inhibitory to the electron transfer system of mitochondria of plants (Taniguchi and White, 1967) or to affect membrane permeability (White and Taniguchi, 1972; Fitts and Hornby, 1978). Sommereyns and Closset (1978) also directly isolated helminthosporal from culture filtrates with diethyl ether and confirmed the chemical characteristics by infrared and ultraviolet spectrometry and biological toxicity. Helminthosporals have also been reported as an antifungal compoud (Nukina and Marumo, 1976; Sommereyns and Closset, 1978).

Some researchers have suggested that helminthosporals act as a phytohormone but not as a phytotoxin (Tamura *et al.*, 1965). Helminthosporal induced elongation of rice seedling shoots in aqueous solutions containing the compound in the concentrations between 10 to 300 ppm. with maximum activity at 50 ppm (Tamura *et al.*, 1963). Briggs (1966) reported that helminthosporic acid stimulated lettuce seedling growth and suggested the possibility of employing this chemicals in the

malting industry, to produce amylase. Nukina *et al.* (1975) analyzed the substances produced by *H. setariae* and *H. sativum* and reported that helminthosporal stimulated the elongation of rice seedlings. Endosperm tests indicated that helminthosporal increased the production of reducing sugars in barley, but the concentration range that was stimulatory was different from that of gibberellic acid (Mander *et al.*, 1975; Turner *et al.*, 1978). The chemical synthesis of gibberellic acid from the helminthosporals has been attempted but without success (Mander and Palmer, 1979; Turner *et al.* 1980).

Gibberellic acid, which causes elongation of plant shoots, has also been reported to inhibit root growth in many plants (Thimann, 1977). Yadav and Mandahar (1981) extracted a cytokinin-like compound from *H. sativum* diseased barley and observed the increase of the reducing sugars in the leaves after he injected this component into barley leaves.

It was hypothesized that D-toxin defined in the previous work might act as gibberellic acid in malting barley plants. These experiments were done to investigate plant response to the diethyl ether soluble toxin of *H. sativum*, to identify the nature of it on the barley.

Materials and Methods

Fungal isolates and preparation of toxins

Helminthosproium sativum isolate ROO2 was obtained and maintained as previously described (Lee, 1988). The toxin (s) obtained from day old shake cultures were separated by passage through a C-18 column, then collected and concentrated in methanol. Diethyl ether was added to the concentrated toxin, and this mixture was chilled for two hrs in the ice water. The mixture was centrifuged at 12,000 g for 10 min and the supernant (containing the compounds soluble in diethyl ether, called "D-toxin") was collected. Lettuce bioassay was conducted for a toxic substance as previously described (Lee, 1988). The toxin extract was resolubilized with 50 u*l* methanol and was mixed mixed with 2 m*l* distilled water for the lettuce bioassay. Ten seedlings were applied for bioassay with a statistic analysis. D-toxin was not highly purified, but was on the process of purifications.

High pressure liquid chromatography

D-toxin was purified by high pressure liquid chromatography (HPLC, Waters Associates Inc., M-6000 A) with a model 660 gradient program running at 30℃ on a reverse phase C-18 column (μB Bondapak), 30 cm×3.9 mm i.d. The eluent was monitored by UV absorbance at 272 nm, which is the maximum peak of helminthosporal in water. D-toxin defined in the previous work (Lee, 1988) was injected on the C-18 column and eluted with a linear gradient of double distilled water to acetonitrile at a flow rate of 2 m*l* per min. The acetonitrile was eluted from 0 to 100% over 20 min. The fraction being toxic to lettuces was collected and dried at 40 C under a mild flow of nitrogen gas. The solvents collected from HPLC were completely evaporated at 40 C under vacuum. D-toxin purified by HPLC was resolubilized with methanol and used for understanding in the further chemical nature of it.

Chemical analysis of D-toxin

D-toxin was analyzed using a gas chromatograph (GC: Hewlett-Packard 5880 equipped with a CPsil-5, 30 meters ×0.32 mm i.d. fused silica column provided by Chrompack. D-toxin esterified or reacted with some chemicals (mentioned later) was then analyzed by GC. The column temperature was isothermal for 2 min at 200 C, then programmed to 250 C at the rate of 2 degrees/min. The samples showing peaks were dissolved in $CDCl_3$ in thin glass tubes and were analysed by proton nuclear magnetic resonance (Jeol, FXRAT 90Q FTNMR). Mass spectra were obtained from a Hewlett Packard 5992 gas chromatograph-mass spectrometer (GC-MS). The temperature was programmed from 140 to 250 C at 10 degrees/min. The ultraviolet absorption spectrum of D-toxin was also determined in methanol and in 0.5% methanol using a Beckman DU-7 spectrometer from 200 to 400 nm. The GC and UV absorbance procedures were used for the steps of the purification to track the D-toxin and the effect of barley on

D-toxin.

Chemical structure of D-toxin

Presence of less volatile compunds was checked to esterify any compound which might be missed (Christie, 1982). Reduction and trimethylsilyl ether derivatives were employed in the chemical confirmation of D-toxin. Sodium borohydride ($NaBH_4$) was used to chemically reduce *oxo* groups to hydroxyl to alcohol groups. The number of *oxo* groups on the molecule can then be determined by the charge in molecular weight. The D-toxin (0.5 m*l*) was reduced into an alcohol form by addition of 20 mg $NaBH_4$ in 1 m*l* of methanol. After 20 min, the sample was diluted with 10 m*l* distilled water and the reduced compound (s) was extracted with a Baker C-18 column and injected into a GC-MS. The original and reduced D-toxin (100 μ*l*) were mixed with two m*l* distilled water and applied to the lettuce bioassay. One m*l* of dimethylformamide (DMF) and ten mg of *n*-butyl boronic acid, which reacts with 1, 2-or 1, 3-*diols*, were added to the reduced, alcohol form of D-toxin to the test for proximity of the hydroxyl groups (Christie, 1982). Products which might be produced by this reaction were determined by GC as previously described. The alcohol form of D-toxin was also reacted with *n, o-bis* (trimethylsilyl) trifluoroacetamide to form the trimethylsilyl ether derivative. The original D-toxin, the reduced D-toxin, and the trimethylsilyl ether derivatives were injected into a GC-MS for characterization of D-toxin.

Endosperm tests for malting barleys

Two malting barley lines (*Hordeum vulgare L.*), 'Dickson' and 'Larker', were employed for endosperm and toxicity tests. D-toxin and gibberellic acid (GA_3, Sigma reagent) were tested at comparable concentrations (Mander *et al.*, 74; Moore, 81: Tamura *et al.*, 1963). For purposes of quantitative estimation, it was assumed that D-toxin was helminthosporal. D-toxin was determined by measuring the gas chromatographic peak areas, which were quantified at 260 nm ultraviolet absorption (Extinction coefficent = 11,000, Merck Index 10th Eds., 1983) for helminthosporal. Barley seeds were surface-sterilized in 30% H_2O_2 for five min. The residual peroxide was eliminated by washing four times with sterile distilled water. All seeds were then soaked for 20hr at 5 C and the embryo part was removed. The four sterilized barley-endosperms per tube were incubated at each of the different concentrations of gibberellic acid or D-toxin at 25 C for two days, based on Moore's gibberellic acid experiment (1981). D-toxin extract and gibberellic acid were dissolved in methanol, mixed with deionized water until the methanol content was 5% (v/v), and applied to barley endosperm tests. The response of the barley endosperms to two chemicals were measured by the amount of reducing sugars released (Miller, 1959). The experiment was duplicated.

Barley responses

Ten barley (*Hordeum vulgare* L.) lines were obtained from Departmet of Agronomy, North Dakota State University, Fargo, ND 58105 and used to test effects of toxins produced by *H. sativum* isolate ROO2. Barley responses were detemined with Davis' method (1985). Two concentrations of toxins, 10 μg (DL) of helminthosporal equavalent. Culture filtrate Equavalent/treatment were tested *in vitro*. The 'low' concentration (DL) was chosen to be near or beyond the dilution end-point as previously described by Lee (1987).

Results

Purification

On the HPLC column, two fractions appeared toxic to lettuce with UV absorbance at 272 nm. The fraction have higher toxic to lettuces was collected and dried for the following chemical analysis; D-toxin purified by HPLC showed the maxinium peaks at 268 and 272 nm at methanol and 5% methanol in UV analysis. Gas chromatography showed only two main peaks at 2.65 and 2.67 min. Both, two compounds indicated in GC had a molecular weight of 234 m/z, but the fragmentation patterns of both in MS-GC showed slightly differences. No additional peak in the D-toxin was found by GC, with or without estification of diazomethane. Proton nuclear magnetic resonance (nmr) showed H nmr ($CDCl_3$, TMS), δ5.17 ppm (s 1H CHO), and δ 5.017 ppm (s, 1H CHO).

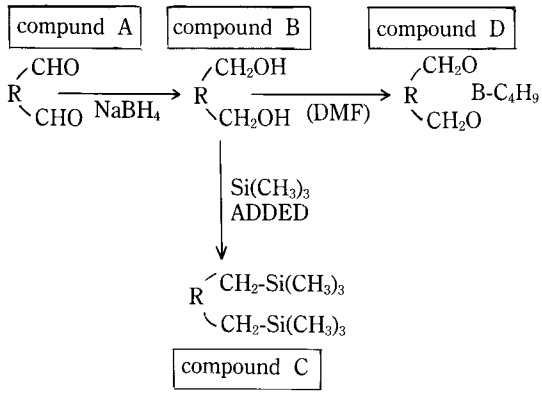

Fig. 1. Flow chart for chemical reactions of D-toxin. The molecular weights of A, B, and C compounmds determined by 234, 238, and 382 m/z in GC-MS, respectively. Lettuce bioassays of A and B compounds indicated 10.1± 1.7 and 47.1± 3.1 mm with 53.4± 2.5 mm as a control, respectively.

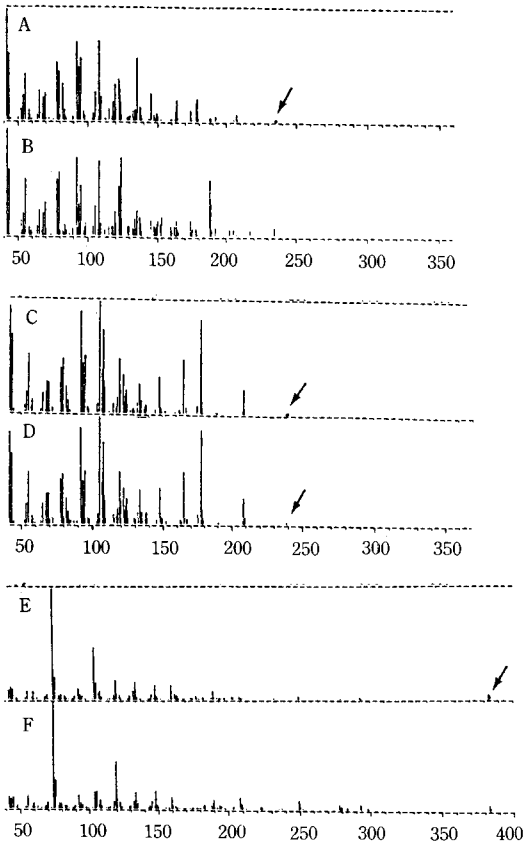

Fig. 2. The molecular weights of the compounds: A and B resulted from the compound A, C and D from the compound B, and E and F from the compound C shown in Fig. 1.

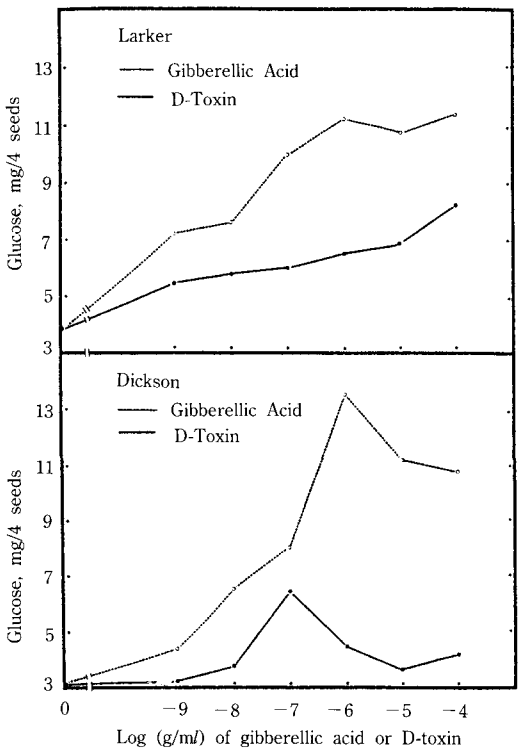

Fig. 3. Effects of D-toxin on α-amylase activity. Each points represented the average values of three experiments with one tube containig four endosperms.

Chemical reactions

The glycol form of D-toxin was detected after reduction and had a molecular weight of 238 which indicated the compound 'B' (Fig. 1). The mass spectra of B is shown at the compounds of C and D in Fig. 2. This indicated that the D-toxin had two aldehyde groups in the molecule. This was consistent with the results of UV and nmr. The trimethylsilyl ether derivatives of D-toxin were detected with a molecular charge of (m/z) 382, as indicated by compound 'C' and 'D' in Fig. 1 and 2. The aldehydes of D-toxin do not have a 1, 2- or 1, 3-spatial arrangement because n-butylboronate derivatives (compound 'D' in Fig. 1 and 2) were not found throughout 40 min running in the chromatograph. The compound 'B' was tested in the lettuce bioassay and was not active.

Barley responses

D-toxin was applied to barley endosperm and

Table I. The root and coleoptile responses of two barley lines, Larker and Dickson, to different concentrations of D-toxin and gibberellic acid. The numbers indicated the length growths (mm) of two malting barley lines at 25 C for three days[a].

Treatments	Lsrkers		Dickson	
	Roots	Coleoptiles	Roots	Coleoptiles
control[b]				
Water	36.5	66.5	35.5	68.3
MeOH	40.0	62.2	35.4	64.2
D-toxin[c]				
10 μg	33.0	47.3	45.7	57.5
2 μg	54.1	59.7	55.3	65.0
0.2 μg	47.3	64.3	45.3	72.3
Gibberellic acid[d]				
10 μg	29.0	79.2	32.7	83.2
2 μg	31.5	72.5	34.0	79.0
0.2 μg	35.5	66.0	34.5	74.5
0.02 μg	34.0	56.7	38.2	67.8

[a]Least significant differences were 2.40 and 4.37 mm at P=0.05 for root and coleoptile lengths, respectively. The root lengths of seedlings were inversely proportional to the coleoptile lengths of seedlings ($R^2=-0.714$). Six seedlings collected for this experiment.
[b]Distilled water and 0.25% methanol applied as the control.
[c]D-toxin measured by the ultraviolet absobance at 268 nm.
[d]Gibberellic acid (GA_3, Sigma reagent grade) used.

Table II. Barley root responses of D-toxin and the field indices of rot (RD) or foliar blotch (FD) made in the field tests[a].

Barley lines	Controls[b]		D-toxin[c]		Field Indices[d]	
	WC	MC	DH	DL	RD	FD
Azure	32.8	40.8	33.8	43.5	2.1	4
Bumper	33.8	44.7	38.3	46.2	2.2	3
Conquest	42.2	36.5	37.7	52.8	2.7	5
Dickson	33.5	44.8	42.7	41.2	2.4	3
Glenn	35.8	41.0	34.0	46.0	2.3	4
Larker	37.5	37.5	34.7	45.7	2.7	7
Morex	31.3	39.5	40.2	50.7	2.6	5
Nordic	42.7	56.0	39.3	47.1	2.1	2
Park	41.5	61.1	50.5	56.0	2.2	3
Robust	37.3	46.7	46.7	49.5	2.7	2

[a]LSD was 1.71 mm at P=0.05.
[b]Water control (WC) and 0.25% methanol (MC).
[c]D-toxin (0.4 Culture Filtrate Equivalent/ml for DH and 0.00625 Culture Filtrate Equivalent/ml for DL).
[d]Field indices of two different diseases of *H. sativum*. Root rot disease (RD) from R.W. Stack, 1986. Common root rot Barley 1980-83 in culture and biological treatments for control of Plant diseases. Amer. Phytopth. Soc. (in press). Foliar disease indices (FD) from V.P. Pederson, Department of Plant pathology, NDSU, Fargo, ND 58105.

the response compared to gibberellic acid. The activities were measured by the amounts of reducing sugar released from barley endosperms. The malting barley line. 'Larker', showed a response to all concentrations of D-toxin and of gibberellic acid and generally increased activity with increased concentration (Fig. 2). Larker's response to D-toxin was approximately half that of gibberellic acid. The response of Dickson was not so straightforward. The response increased until a peak value was reached (10^{-7} for D-toxin, 10^{-5} for gibberellic acid), and then decreased with further increase in concentration (Fig. 3). Barley growth responses to D-toxin and gibbeellic acid were determined (Table I). Gibberellic acid never stimulated root growth and only slightly reduced root growth in Larker but not in Dickson. D-toxin stimulated root growth in both lines at 2 μg and inhibited root growth in Larker at 10 μg but not Dickson. Gibberellic acid stimulated coleoptile growth in both lines with increased concentrations. D-toxin never stimulated the coleoptile growth and inhibited it at the three concentrations shown in Table I. Linear regression of root to coleoptile responses to gibberellic acid was done with $R=-0.71$ ($P<0.05$) but that to D-toxin did with $R=-0.001$ ($P>0.05$). Biological action of gibberellic acid did stimulate the shoot growth, but D-toxin did not.

Effects of D-toxin on barleys

Ten barley lines were tested for the response to D-toxin (Table II and Fig. 4). The lines showed different responses to high concentrations of D-toxin. some showing stimulation, some inhibition and some no response. Analysis of variance showed a significant treatment by the barley line interaction term. The responses of the ten lines to D-toxin were compared to known reactions of those lines for root disease (Common root rot;

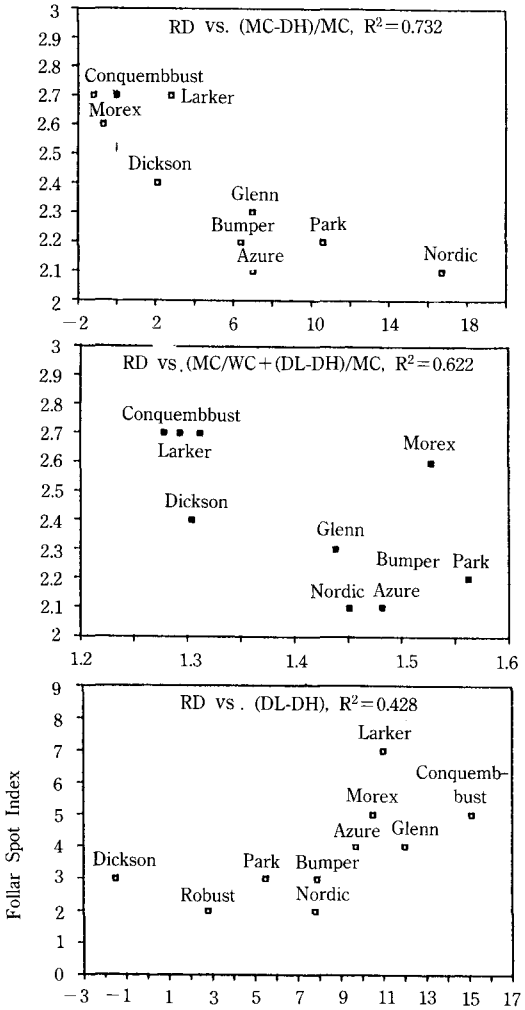

Fig. 4. Correlations of the two field respones of *H. sativum* diseases, root and foliar diseases with the responses of D-toxins, each terms employed here comes from Table II.

R. W. Stack, personal communication) and for foliar disease (spot blotch; V. P. Pederson, personal communication). When a single variate was used, only one measurement, (MC-DH), was significantly correlated to root rot reaction with $R^2=0.732$, $P<0.05$. The correlations, (DL-DH)/MC, were significantly improved with $R^2=0.0.622$ $P<0.05$), when a second term correction for methanol response (MC/WC) was added (Table II and Fig. 4). Only one variate, (DL-DH), was significantly correlated to foliar disease reaction. This correlation was not improved by any combination with other variates.

Discussion

Chemical nature of D-toxin

The HPLC reverse phase C-18 column indicated that the D-toxin extracted from diether ether was a highly purified one. The chemical nature of D-toxin was similar to that the compounds extracted from diethyl ether with nmr and UV (Sommeyern and Closset, 1978), and helminthosporal or its related compounds with GC-MS and functional group (-CHO)(DeMay *et al.*, 1961; 1963; Mander *et al.*, 1974; 1979; Tamure *et al.*, 1963; 1965). Based on the above, it was concluded that this D-toxin should be a helminthosporal or its closely related compound, since all values agreed with published values for helminthosporal. We were unable to obtain a sample of helmithosporal for other workers for comparison and to compare each others.

We tried to separate the two compounds showed in GC analysis with Thin Layer Chromatography and other HPLC columns, which were not described in this papaer, but did not separate D-toxin purified by HPLC. Here, D-toxin was, if assumed that it would be a polymer of helminthosporals with a heat unstable bond, speculated to be detected by two compound in GC analysis. This speculation agreed with Nukina's proposal (1975 ; 1976), *cis*-sativendiol, a polymer of two helminthosporals, but the result of DMF treated D-toxin disagree with a *cis* formation. GC detection of D-toxin was done with heat treatment. Heat treatment of D-toxin showed loss of toxicity in lettuce. It was speculated that D-toxin should be a polymer of two helminthospoals with a heat unstable bond.

Biological effects of D-toxin

Since helminthosporal corresponds to the 'C' and 'D' rings of gibberellic acid, it may have effect similar to gibberellic acid on plants (Tamura *et al.*, 1963 ; 1965; Mander *et al.*, 1974 ; 1979). This expectation was made, as based on rice seedling and barley endosperm tests, and proved correct for stimulatation of reducing sugar in malting

barley endosperms. The responses of D-toxin on barley roots and coleoptiles were completely different from those of gibberellic acid. Several workers who considered helminthosporal as a gibberellic acid-like compound did so on basis of a single tests in each case. It might be speculated that D-toxin was not as biologically same as gibbeellic acid at roots and coleoptiles in barley systems. Linear analysis for root to coleoptile responses shown in Table II revealed that GA_3 stimulates the coleoptile growth but that inhibit the root growoth. D-toxin also inhibit the root growth and stimulate productivity of the reducing sugar like GA_3, but not stimulate the coleoptile growth.

The resistant line, Dickson, showed the restricted range in endopserm test. D-toxin directly affected the foliar disease and indirectly the root rot disease in malting barley lines. The variate, MC /WC, was speculated to be an environmental effects of individual lines in methanol solution. These correlations shown in Fig. 4. seemed to indicate that D-toxin should be related to the disease progress in barley. It was specultated that D-toxin, a polymer of two helminthosporal, should be involved in plant metabolism and help fungal metabolism in the interaction between pathogen and host.

Based on the upper experiment, it was concluded that D-toxin is similar to giberellic acid in several regardings; all compounds produced by the plant pathogenic fungi, inhibition of root growth in barley, and increase of reducing sugar in barley endosperm tests. However, D-toxin is not similar to giberellic acid in this regardings; increase of reducing sugar at the different concentrations of each compound, chemical structures, and no stimulation of shoot growth by D-toxin. It was not concluded what D-toxin is, but concluded that D-toxin is a kind or polymer of helminthosporal. The D-toxin produced by the fungus would be related to the plant hormone, gibberellic acid, and also involved in the two kind of disease of barley with host specific term.

摘 要

*H. sativum*에서 분리되고 명명된 D-toxin은 UV, GC-MS, 및 화학분석결과 helminthosporal와 비슷한 결과를 얻었으나, helminthosporal와 같지는 않았다. 이 식물독물질은 barley endosperm실험에서 gibberellic acid와 같은 결과를 반응을 나타내었으나, 보리뿌리 및 줄기반응에서 gibberellic acid와 같지가 않았다. 또한, 이 물질은 통계처리결과 은 반응결과를 Host specific disease로 알려진, Foliar spot and Root rot diseases에 작용하는 것으로 나타났다.

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