韓 國 菌 學 會 誌 第16卷 第1號
Kor. J. Mycol. Vol. 16, No. 1, 9-15, 1988

Separation and Purification of two toxins produced by *H. sativum* P. K. & B.

Sang Sun Lee*, Brady A. Vick and Robert W. Stack.**

Department of Biology Education, Korean National University of education, Chong Puk 420-23, Department of Plant Pathology, and USDA in North Dakota State University, Fargo, ND 58105***

*H. sativum*이 生成하는 植物 독소물질 分離

이상선*·브레리 빅·로버트 스택**

한국교원대학교 생물교육학과*·미국노스타코타대학 식물병리학과·와 유에이디직원**

ABSTRACT: Two kinds of toxins were demonstrated in the culture filtrate of *H. sativum*, and were called 'M' and 'D' toxins. The lettuce bioassay indicated that D-toxin caused less root growth inhibition than M-toxin. Chemical analysis indicated that M-toxin was a very unusual small peptide. D-toxin was shown to have chemical characteristics similar to helminthosporal based on ultraviolet, proton nuclear magnetic resonance and mass spectra. D-toxin was composed of at least two isomers.

KEYWORDS: *Helminthosporium sativum*, toxin, toxic metabolites, D-toxin, M-toxin, Helminthosporal.

Helminthosporium sativum Pamm., King & Bakke (*Cochliobolus sativus* (Ito & Kurib) Drechsler ex. Dastur) is an important pathogen of spring wheat and barley in the spring grain areas. It attacks all plant parts causing leaf spots, blights, and root and crown rots. While these diseases have been intensively studied, little is known of the mechanism(s) of pathogenesis. *H. sativum* produces toxins in liquid culture(Davis, 1983) and these have previously been investigated for a possible role in the disease, but with inconclusive results(Pringle, 1976a; 1976b).

It has been proposed that *H. sativum* is evolutionarily related to *H. victoriae* because they produce similar toxin compounds, 'victoxinine' and 'helminthosporal'(Pringle 1976a). Nelson(1959; 1960) found the teleomorphs of *H. carbonum* and *H. victoriae*, artificially crossed the progeny and studied them for toxin production(Nelson *et al.*, 1963). All species of *Helminthosporium* mentioned above share the same teleomorph genus, *Cochliobolus* (Luttrell, 1963; 1964; Shoemaker, 1959). *H. sativum* would be closely related to *H. carbonum* and *H. victoriae*, which produce cyclopeptide toxins (Kawai *et al.*, 1983 ; Liesch *et al.*, 1982; Pringle and Scheffer, 1964).

At least two kinds of toxin were predicted by experiments with culture filtrates of *H. sativum*(Grayed and Naguib, 1962). Ludwig(1956) and Pringle(1979) made similar proposals based on their observation of symptom patterns in infected plants.

The presence in *H. sativum* culture filtrates of a substance toxic to barley and wheat was reported by Ludwig(1956; 1957), and was first chemically characterized by DeMayo(1961; 1963), who called it 'helminthosporal'. Helminthosporal and related compounds were isolated from culture filtrates, but were considered as phytohormones by Tamura(1963) and Mander(1979). In contrast, helminth-

* 이 논문은 1987년도 문교부 자유공모과제 학술연구조성비에 의하여 연구되었음.

osporal was considered to be a toxin by Sommereyns(1978). The presence of a cytokinin-like compound had been suggested in *H. sativum* infected barleys(Yadav and Mandanar, 1981). A translocation sink was also proposed to be induced by *H. sativum*(Yadav and Mandanar, 1981).

This work was done to separate the toxin(s) produced by and to clarify the nature of toxin(s) produced by *H. sativum*.

Materials and Methods

Culture, extraction and bioassay

H. sativum isolates, ROO2 and F-23, were obtained and maintained(Davis, 1983). The toxin was obtained from filtrates of four days cultures and harvested by elution through a C-18 column(Lee, 1987). Lettuce bioassay was conduced as previously described(Lee, 1987). One unit of culture filtrate equivalent(CFE) equals the amount of toxins present in one ml of the original culture filtrate.

Separation of toxic components

Twenty CFE of C-18 prep were evaporated to dryness at 40℃ under mild vacuum and then 100 ul ultra-pure methanol was added. Five ml of diethyl ether was added and this mixture was chilled for two hr in ice water. The mixture was centrifuged at 12,000×g for 10 min and a dark brown precipitate separated. The supernatant(containing compounds soluble in diethyl ether) was collected. The precipitate was washed several times with diethyl ether, the washing being added to the supernatant. The precipitate was dried under mild vacuum at 40℃ and dissolved in methanol; this fraction was called 'M-toxin'. The combined diethyl ether supernatants were dried, and redissolved in methanol and called 'D-toxin'. Fig.1 is the flow chart for the separation of *H. sativum* toxic components. The values of lettuce bioassay were average of ten replications.

Purification by Sephadex and thin layer Chromatography

A Sephadex G-25(Pharmacia Fine Chemicals, 800 Centenial Av. Piscataway, N.J.) gel was employed for the characterization of the methanol soluble toxin. The Sephadex G-25 was packed in a 2.6 cm×40 cm glass column and washed with distilled water at a continuous flow rate of 5 m*l*/min. One ml methanol solution containing 20 CFE was applied the top of column. The column eluent was collected in five ml samples using a fraction collector(Eldex Fraction Collector, MINI II). From each 5 m*l* fraction, a 2 m*l* aliquot was tested for lettuce root growth response and a 0.5 m*l* aliquot for ninhydrin reaction. This experiment was repeated twice. Fractions showing high toxicity in the lettuce bioassay were concentrated by passage through a C-18 column and ether precipitation as previously described. For analytical purposes, this purified, concentrated M-toxin was reapplied to a Sephadex G-25 column and the eluent fractions tested by bioassay as before. Molecular weight of M-toxin was estimated by comparing its elution pattern with that of vitamin B_{12}(Molecular weight 1300). The determined value was also compared with the standard chart for Sephadex G-25(Janson, 1967). The fractions showing highest toxicity were collected for peptide analysis. The amino acid content of M-toxin was measured with a modified ninhydrin reagent after alkaline hydrolysis (Colowich and Kapnan, 1967). To determine amino acid composition, M-toxin was hydrolyzed in 6 N HCl for 24 hr at 100℃. Amino acids were separated by thin layer chromatography(TLC) (solvent system;n-butanol:acetic acid:H_2O=4 : 1 : 1) on silica gel plates(EM reagents, Cat. 5506) for one hr. The separated amino acids were detected using ninhydrin reagent(Stahl, 1969).

High pressure liquid chromatography

D-toxin was purified by high pressure liquid chromatography(HPLC, Waters Associates Inc., M-6000A) with a model 660 gradient program running at 30℃ on a reverse phase C-18 column(mB Bondapak), 30 cm×3.9 mm i.d. The 100 μl methanol solution containing five CFE was injected through a reverse column. The eluent was monitored by UV absorbance at 272 nm, which is the maximum peak of helminthosporal in water. The eluent was collected in two ml fraction tubes. Each fraction was tested by lettuce bioassay. In a preliminary test, it was determined that acetonitrile inhibited the lettuce root growth more than D-toxin; therefore, the solvents collected from HPLC were completely evaporated at 40℃ under vacuum. The toxin extract was resolubilized with 50 μl methanol and was mixed with two ml distilled water for the lettuce bioassay. The fraction(s) from the silica column which exhibited high toxicity were injected

into a reverse phase C-18 column on the HPLC for further purification. The aliquot was injected on the reverse phase 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. This experiment was repeated twice. The two fractions of D-toxin obtained from 14 and 16 min peaks in reverse phase C-18 column were dried at 40℃ under a mild flow of nitrogen gas.

Chemical analysis of D-toxin

The diethyl ether soluble toxin was analyzed using a gas chromatograph(Hewlett-Packard 5880 equipped with a CPsil-5(30 meters×32 mm i.d.) fused silica column(Chrompack)). The column temperature was isothermal for 2 min at 200℃, then programmed to 250℃ 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, FXRAY 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℃ 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.

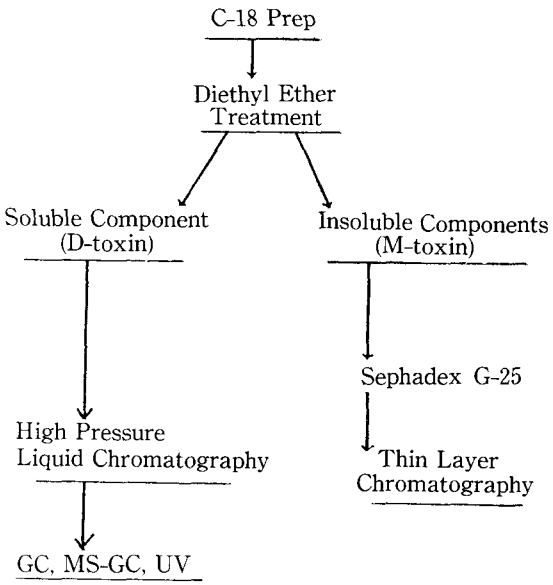

Fig.1. Flow chart for separation of *H. sativum* toxins.

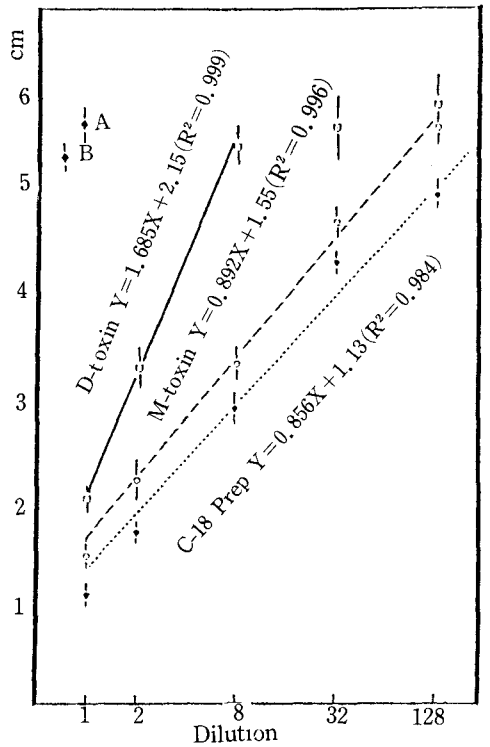

Fig.2. The lettuce responses of C-18 prep, D-toxin and M-toxin with dilutions. Regression model is Y(lettuce resonse, cm)=Log(Dilution rate)+b. All lines correlated at $P<0.05$. A and B indicated distilled water control and 0.25% methanol control, respectively.

Results

Separation of two toxins

Two toxins were separated as shown in the flow chart(Fig.1). Fig.2 showed the responses of the lettuce bioassay for separated toxins(One CFE was applied). D-toxin showed a steeper slope than M-toxin and reached its dilution end-point at 7.9 unit; M-toxin did not reach its dilution end-point until 97 units. The original C-18 preparation(called "C-18 prep") shows a dilution end-point of 192 units. The slope of the M-toxin dilution curve was very close to that of the original C-18 prep.

Diethylether insoluble toxin('M-toxin')

The root length in the lettuce bioassay and the amino acid content of the five ml fractions collected from the Sephadex G-25 column are shown in Fig.3. The amounts of amino acid were determined by ninhydrin based on a standard curve of glutamic acid. The peak of amino acid content was

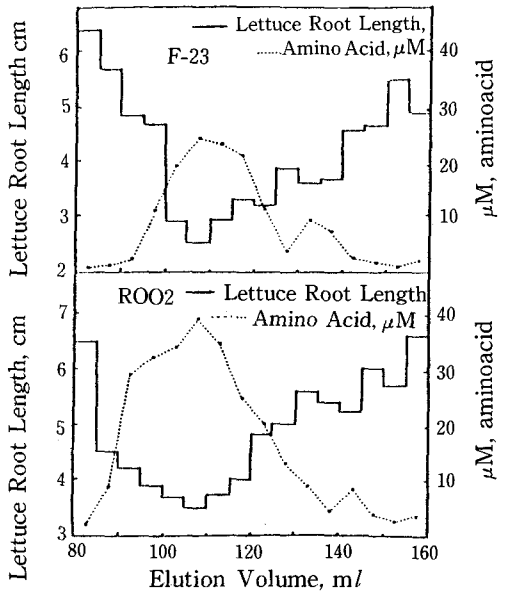

Fig.3. Lettuce root responses and amino acid concentration of M-toxin component eluted through Sephadex G-25(5 m*l* fraction).

located in the same fractions which showed maximum activity in the lettuce bioassay. The fraction showing both the maximum amino acid content and highest toxicity was v_e(105-110 m*l*, k_{av}=.22 to .25), representing 1,500 to 1,800 daltons molecular weight as based on the analysis of a known compound and molecular weight chart. Results with the culture filtrate from isolate F-23 were similar to those of isolate ROO2. At least eight spots of amino acids were detected by thin layer chromatography of the hydrolyzed M-toxin. This indicated that M-toxin was composed of at least eight amino acids. The exact amino aicd composition was not determined in these experiments.

Purification of D-toxin

On the HPLC reverse C-18 column, two fractions showed toxicity to lettuce and UV absorption at 272 nm(Fig.4). These fractions were collected for further analysis. The fractions corresponding to

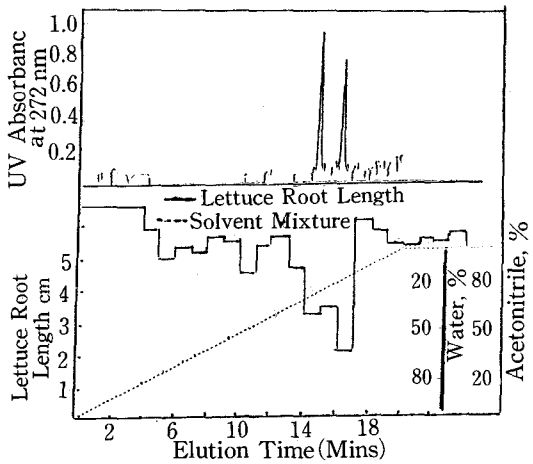

Fig.4. Ultraviolet absorbance and lettuce root responses of D-toxin eluted through the reverse phase C-18 column on high pressure liquid chromatography.

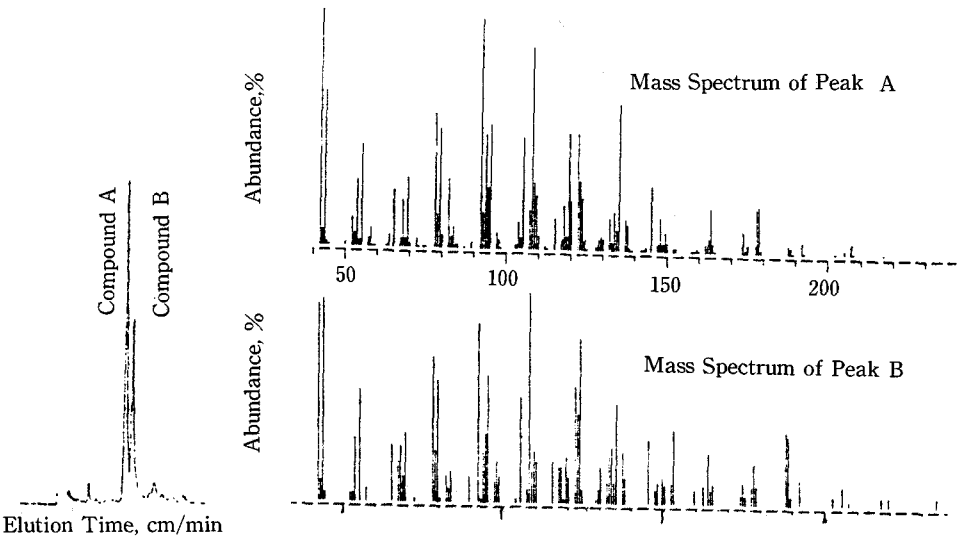

Fig.5. Gas chromatography and mass spectra of D-toxin.

these peaks were toxic in the lettuce bioassay(Fig. 4). These peaks separated by the HPLC reversed phase column were designated 'helminthosporal I and II'. Gas chromatography of these two fractions from the HPLC reverse C-18 column revealed that each showed the same two GC peaks at 2.56 and 2. 67 min('A' and 'B' compounds shown in Fig.5). The two components being separated by GC, therefore, were not the same as the two being separated by HPLC(Fig.4). The GC peaks of both HPLC fractions(I and II) were identical to those of the D-toxin preparations. Neverless, the ratio of A to B compound were not same in GC analysis of I and II compounds separated by HPLC.

Diethyl ether soluble toxin(D-toxin)

Both 14 and 16 min fractions of purified D-toxin(I and II compounds resigned in the above) had a maximum UV absorbance at 268 nm in methanol and 272 nm in 5% methanol. The II compound shown in Fig.4 was employed for the further analysis, nmr and mass spectra. Mass spectra of the two GC peaks at elution times of 2.56('A') and 2.67('B') min indicated that both corresponded to compounds having a molecular weight of 234 daltons(Fig.5). The fragmentation pattern of the two compounds('A' and 'B') in MS-GC showed slightly differences. Mass spectra of two main peaks showed molecular ions at m/z 234 for both peaks. Proton nuclear magnetic resonance(nmr) showed; H nmr($CDCl_3$ TMS) delta, 9.53(s, 1H CHO) and 9.63(s, 1H CHO). Mass spectrum, proton nmr and UV absorption spectrum are consistent with identification of D-toxin as helminthosporal (Tamura *et al.*, 1963; Windholz and Budavari, 1983).

Discussion

Separation of toxins

It was shown that at least two different toxins are produced by *H. sativum*. When the toxicity of each component was calculated and dilution end-points determined, the toxin units of D-toxin and M-toxin added together should be the same as the toxin units for C-18 prep(Fig.2). This was not so; the units of toxicity of D-toxin and M-toxin together accounted for only 55% of the activity of the C-18 prep. Effects of each toxin on the lettuce bioassay were not additive in nature. There appears to be a synergistic effect. The dilutions of each toxin showed different slopes in the lettuce bioassay responses(Fig.2). The biological effects of C-18 prep, and presumably therefore of the culture filtrate, mainly resulted from the presence of M-toxin, especially at the lower concentrations. The effect of unbalanced mixtures of D-toxin and M-toxin were not studied, but might provide useful information about their physiological interaction.

M-toxin

The M-toxin found here for the first time was unusual; because although it was a peptide, it was soluble in methanol. The discovery of peptide M-toxin was consistent with that of cyclopeptide toxins in *H. victoriae*(Pringle and Braun 1957; Pringle and Scheffer, 1964; Walton *et al.*, 1982), *H. carbonum*(Pringle and Braun 1957; Tamura *et al.*, 1963) and *Pyrenophora teres*(Smedegard, 1977). Presence of this peptide toxin in *H. sativum* cultures has been overlooked because previous methods used have removed it. Pringle(1976b; 1979) eliminated this M-toxin by n-butyric alcohol extraction, which has been employed for purification of victorin and HC-toxin. Sommereyns(1978) also eliminated M-toxin by using diethyl ether extraction. Occurrence of a peptide toxin was predicted by Grayed(1962) based on their analysis of nitrogen content of *H. sativum* culture broth.

Based on the average molecular weight of amino acids(150 daltons per amino acid), some of the eight amino acids detected by thin layer chromatography were probably repeated in the primary sequence of M-toxin, It was that there are probably at least 10 amino acids present based on a molecular weight of 1500(spots in TLC work). Since *H. sativum* is closely related to *H. victoriae* and *H. carbonum*(all have *Cochliobolus* teleomorphs), it might be expected that their toxins would be similar in nature. M-toxin was not detected by the ninhydrin reaction until after NaOH hydrolysis, suggesting that M-toxin might be a cyclopeptide compound like victorin or HC-toxin.

D-toxin

The diethyl ether soluble toxin(D-toxin) isolated in this research was similar to diethyl ether extracted compounds described by Sommeyerns(1978). The C-18 column and diethyl ether precipitation employed here removed contaminating compounds and isolated D-toxin with a high degree

of purity(Fig.1). Based on UV absorption spectra(258 nm), proton nmr spectra(two CHO's) and molecular weight(m/z 234) (Fig.5), it was concluded that this D-toxin must be helminthosporal or a closely related compound, since all these values agreed with published values for helminthosporal(DeMayo *et al.*, 1961; 1963; Sommereyns and Closset, 1978; Tamura *et al.*, 1963; 1965). We were unable to obtain a sample of helminthosporal from other workers for comparison.

The HPLC reverse phase C-18 column was found to separate D-toxin components better than the silica or alumina employed by previous researchers(DeMayo *et al.*, 1961; 1963; Smedegard, 1977; Tamura *et al.*, 1963). Heat treatment was not employed in purification of D-toxin in this experiment. It had been previously shown that heat treatment destroyed toxic activity(Lee, 1987). Helminthosporal A and B corresponding to peaks at 2.56 and 2.67 min on GC and the same molecular weight, 234 daltons. Based on the reported chemical structure of helminthosporal(DeMayo *et al.*, 1961; 1963; Tamura *et al.*, 1963; 1965), the theoretical ultraviolet absorption spectrum could be calculated(Pavia *et al.*, 1979; Silverstein *et al.*, 1981). The calculated peak UV absorbance should be 266 nm. The maximum absorption wavelength of D-toxin was 268 nm, close to the value of helminthosporal. In the mass spectrometer, the patterns of fragmentation of helminthosporal A and B had similar characteristics, but were not identical. The mass spectra of both helminthosporal A and B indicated a molecular weight of 234 daltons. Thus it appears likely that the two compounds(helminthosporal A and B) separated by GC are geometric or stereoisomers of helminthosporal. The I and II compounds seemed to be a polymer of A and B compounds, but they were not same with a different bond(A-B/B-A) or a site. Also, the I and II compounds were considered to be easily degraded with heat processes.

摘 要

H. sativum 배양액에서 두가지 종류의 식물독소물질이 검출되었으며, 이들을 M-toxin과 D-toxin으로 명명하였다. Lettuce 식물반응결과는 D-toxin 보다 뿌리성장의 저해작용이 적었다. 화학분석결과 M-toxin은 특별히 작은 peptide로 되어 있었다. D-toxin는 Helminthosporal와 화학적 성질이 비슷하였으나 같지는 않았다. 이는 UV, proton NMR와 질량분석기의 기본 자료인 결과이며, D-toxin은 적어도 두개의 isomers로 되어 있었다.

References

Colowich, S.P. and Kapnan, N.D.(1967):Methods in enzymology. Vol II. Enzyme structure. Academic Press, N.Y. and London, see pg 325-329.

Davis, C.M. (1983): *Helminthosporium sativum* P. K. & B. Toxic metabolites: Wheat cultivar response and lettuce bioassay. M.S. Thesis, North Dakota State University, pp.86.

DeMayo, r., Spencer, E.Y. and White, R.W.(1961): Helminthosporal, the toxin from *Helminthosporium sativum* I. Isoltion and Characterization. *Can. J. Chem.* **39:** 1608-1612.

DeMayo, P., Spencer, E.Y. and White, R.W.(1963): Terpenoids. IV. The structure and stereochemistry of Helminthosporal *Can. J. Chem.* **41:** 2996-3004.

Grayed, S.K. and Naguib, K.(1962):The effect of pH values on the toxicity of culture filtrates of *Helminthosporium sativum* and its possible corelationships to extracellular nitrogen accumulation. *Mycopathol. Mycol. Appl.* **16:** 83-89.

Janson, J. (1967): Absorption phenomena on Sephadex. *J. Chromatog.* **28:** 12-20.

Kawai, M., Rich, D.H. and Watlon, J.D.(1983): The structure and conformation of HC-toxin. *Biochem. Biophys. Res. Comm.* **111:** 398-403.

Lee, S.S.(1987):Measurement of Dilution end-point and phytotoxicity of toxic metabolites produced by *H. sativum* in Barley, wheat and Lettuce roots. *Korean J. Plant Pathology* **3:** 198-202.

Liesch, J.M., Sweeley, C.C., Staffeld, G.D., Anderson, M.S., Weber, D.J. and Scheffer, R.P.(1982): Structure of HC-toxin a cyclic tetrapeptide from *Helminthosporium carbonum. Tetrahedon* **38:** 45-48.

Ludwig, R.A.(1957):Toxin production by *Helminthosporium sativum* P.K. & H. and its significance in disease development. *Can. J. Botany* **35:** 291-303

Ludwig, R.A., Clark, R.A., Julin, J.B. and Robinson, D.B.(1957): Studies on seedling diseases of barley caused by *Helminthosporium sativum* P.K. & B. *Can. J. Botany* **34:** 653-657.

Luttrell, E.S. (1963): Taxonomic criteria in *Helminthosporium. Mycologia* **55:** 643-647.

Luttrell, E.S.(1964):Systemics of *Helminthosporium* and related genera. *Mycologia* **56:** 119-132.

Mander, L.N. and Palmer, L.P.(1979): Synthetic plant growth regulators. IV. the preparation of hydroxylated helminthosporic acid analogues. *Aust. J. Chem* **32:** 823-832.

Nelson, R.R. (1959): *Cochliobolus victoriae* the perfect stage of *Helminthosporium carbonum. Phytopathology* **49:** 807-810.

Nelson, R.R.(1960): *Cochliobolus victoriae,* the perfect stage of *H. victoriae. Phytopathology* **50:** 774-784.

Nelson, R.R.,Scheffer, R.P. and Pringle, R.B.(1963): Genetic control of toxin production in *Helminthosporium victoriae. Phytopathology* **53:** 385-388.

Paiva, D.L., Lampman, G.M. and Kriz, Jr, G.S.(1979): Introduction to spectrometry. 367 pp illustr. Saunders College. Philadelphia, see pg 198-205.

Pringle, R.B. (1976a) Comparative biochemistry of the phytopathogenic fungus *Helminthosporium.* XVI. The production of victoxinine by *H. sativum* and *H. victoriae. Can. J. Biochem.* **54:** 783 -787.

Pringle, R.B. (1976b) Role of toxins in etiology of spot blotch disease of wheat. *Can. J. Botany* **55:** 1801-1806.

Pringle, R.B.(1979): Role of toxins in etiology of spot blotch disease of barley. *Can. Plant. Dis. Surv.* **59:** 74-79.

Pringle, R.B. and Braun, A.C.(1957):Constitution of the toxin of *Helminthosporium victoriae. Nature* **111:** 1206-1207.

Pringle, R.B. and Scheffer, R.T.(1964): Isolation of the host specific toxin and a related substance with nonspecific toxicity from *H. carbonum. Phytopathology* **57:** 1161-1172.

Shoemaker, R.A.(1959): Nomenclature of Drechslera and *Bipolaris* grass parasites segregrated from *'Helminthosporium'. Can. J. Botany* **37:** 879-889.

Silverstein, R.M., Bosseler, G.C. and Morrill, T.C. (1981): Spectrometric identification of organic compounds. 4th ed. John Wiley & Sons. N.Y. pp. 442.

Smedegard, V.P. (1977): Isolation of two toxins produced by *Pyrenophora teres* and their significance in disease development of net-spot blotch on barley. *Physiol. Plant Pathology* **10:** 203-211.

Sommereyns, G. and Closset, J.L.(1978): Preparation of helminthosporal by extraction with diethyl ether fro culture filtrates of *H. sativum* P.K.B. *Phytopath. Z.* **92:** 202-210.

Stahl, E.(1969):Thin layer chromatography. 2nd ed. Springer-Verlag. N.Y. see pg 730-786.

Tamura, S., Sakurai, A., Kainuma, K. and Takai, M.(1963): Isolation of helminthosporal as natural plant growth regulator and its chemical structure. *Agr. Biol. Chem.* **27**: 738-739.

Tamura, S., Sakurai, A., Kainuma, K. and Takai, M.(1965): Isolation of helminthosporal as a natural plant growth-regulator and its chemical structure. *Agri. Biol. Chem.* **29**: 216-221.

Walton, J.D., Earlie, E.D. and Gibson, B.W.(1982): Purification and structure of the host specific toxin from *Helminthosporium carbonum* race I. *Biochem. Biophys. Res. Comm.* **107:** 785-794.

Windholz, M. and Budavari, S.(1983): The Merck index. Merck & Co., Inc. Rahway, N.J., pp.1436, see pg 669.

Yadav, B.S. and Mandanar, C.L.(1981). Secretion of cytokinin-like substances *in vivo* and *in vitro* by *Helminthosporium sativum* and their role in pathogenesis. *J. of Plant Dis. & Protection(India)* **88:** 726-733.

Accepted for Publication 28 January