TOXIC COMPOUNDS PRODUCED BY AN OBLIGATE PARASITE *SCLEROSPORA GRAMINICOLA* (Sacc.) Schroet

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TOXIC COMPOUNDS PRODUCED BY AN OBLIGATE PARASITE *SCLEROSPORA GRAMINICOLA* (Sacc.) Schroet.

By

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CERTIFICATE

This is to certify that the thesis entitled "TOXIC COMPOUNDS PRODUCED BY AN OBLIGATE PARASITE SCLEROSPORA GRAMINICOLA (Sacc.)Schroet." submitted by Mr. Suhas P. Wani for the degree of DOCTOR OF PHILOSOPHY IN AGRICULTURAL MICROBIOLOGY of the University of Agricultural Sciences, Bangalore, is a record of research work done by him during the period of his study in this University under my guidance and supervision, and the thesis has not previously formed the basis for the award of any degree, diploma, associate-ship, fellowship or other similar titles.

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INTRODUCTION

I. INTRODUCTION

Pearl millet or bajra (Pennisetum typhoides (Burm.f) Stapf and Hubb is one of the common millets that serve as foodgrains in India. Pearl millet plant belongs to the family Poaceae. It's grains are used for human consumption and plant stalks are fed to cattle. In India, it covers an area of about 973 million hectares. It comes up well in any kind of soil and for this reason poor soils not fit for growing rice, wheat and sorghum usually are selected for this crop. It is grown extensively in Gujarat, Karnataka, Maharashtra, Madhya Pradesh, Rajasthan, Tamil Nadu and Uttar Pradesh. Although about twenty diseases of this crop are known, the green ear or downy mildew disease caused by Sclerospora graminicola (Sacc.) Schroet, is one of the most important and widespread diseases in India.

Sclerospora graminicola (Sacc.) Schroet, belongs to the class : Phycomycetes, order : Pernosporales and family : Peronosporaceae. It is an obligate parasite on it's host. Downy mildew of pearl millet was first reported by Butler in 1907 from India. The name green ear explains the transformation of the ear, wholly or partially, into a vegetative structure composed of a mass of green twisted leaf-like structures. The fungus causes various types of symptoms in bajra. The disease appears at all stages of development of the plant, starting from the seedling

stage upto the ear stage. Systemic infection is observed early in the seedling stage when the plants attain 5-15 cm height. Systemically diseased plants are significantly stunted with chlorotic leaves which sporulate heavily. In the old plants yellowing and whitening of leaves are observed. As the disease progresses, the chlorotic leaves turn yellowish brown and finally reddish brown. Depending upon the severity of the disease, the young infected plants either dry up or rarely grow further and produce earheads. Dwarfing and discolouration lead to the early death of the infected plants. Frequently, plants having foliage free of other symptoms develop infected spikes. Sometimes, normal earheads develop on diseased plant.

This disease causes a severe damage to bajra crop resulting in marked loss in grain yield. Mitter and Tandon (1930) reported 45 per cent loss owing to the green ear disease, near Allahabad in India. An extensive survey of bajra crop in Rajasthan (Mathur and Dalela, 1971) during 1962 and 1964, revealed that the disease incidence in different fields range from 0-27 per cent. On the basis of data collected in 1962 and 1964, the monetary loss owing to the green ear disease in Rajasthan alone was estimated to be more than 20 million rupees every year. It is estimated (Anonymous, 1971) that the average annual loss in high yielding bajra varieties due to the downy mildew infection was about 30 per cent in India.

Eventhough, this disease causes such a devastating damage to bajra crop, till now there is no proper control measure except using resistant varieties of host plant. If the exact mechanism of symptoms causation in bajra plant by S.graminicola fungus is understood certainly, it will help in suggesting good control measures and also a way to screen large number of resistant varieties against downy mildew may be found out. The objectives of this study were:

1) Isolation of a toxic compound from the S.graminicola infected bajra plants.

2) To find out the role of the toxic compound in causing the symptoms in plants.

3) To understand the mechanism of action of the toxic compound in its host.

4) To elucidate the biological properties of the toxin.

5) To purify and chemically characterize the toxic compound.

6) To study physical and biochemical properties of the toxic compound.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

The idea that plant pathogenic organisms may produce toxic substances which play a causal role in plant diseases was reported as early as 1886. de Bary (1886) while working with Sclerotinia sclerotiorum on carrots succeeded in reproducing soft-rot by applying a sterile extract from rotten carrots to healthy carrot tissues.

Gottlieb (1943), Braun (1950) and Dimond and Waggoner (1953) reported that the metabolites produced by the plant pathogens are capable of producing disease symptoms similar to those found in nature. These metabolites were produced by the pathogens both in vivo and in vitro and are listed in the reviews by several workers (Wheeler and Luke, 1963; Pringle and Scheffer, 1964; Kalyanasundaram and Charudattan, 1966; Wheeler and Hanchy, 1968; Wright, 1968; Owens, 1969; Samaddar, 1970; Patil, 1974; Strobel, 1974a and 1977; and Rai, 1977a).

In the field of plant pathology the word toxin has been used under varied contexts that its significance has become difficult to estimate. Terms such as low molecular weight toxins, high molecular weight toxins, fungal toxins, bacterial toxins, mycotoxins, phytotoxins, plant toxins, vivotoxins, pathotoxins, host-specific toxins, primary and secondary determinants have been used by several authors. Dimond and Waggoner (1953) discriminated between "toxins" and "vivotoxins". A toxin was

defined as "any compound produced by a microorganism which is toxic to plants" and a vivotoxin as "a substance produced in the infected host by the pathogen and/or its host, which functions in the production of disease, but is not itself the initial inciting agent of disease". Later, Dimond (1955) stated that a substance can be regarded as a vivotoxin if it meets the following criteria -

1. It could be isolated from the diseased plant but not be present in the healthy host;

2. It could be chemically characterised; and

3. When introduced, in pure form, into a healthy host, it must produce the symptoms of disease or a portion of a syndrome.

Wheeler and Luke (1963) proposed the terms "pathotoxin" and "phytotoxin". Pathotoxins are those toxins which play an important role in disease production. They listed four criteria to prove a toxin to be pathotoxin. They are:

1. The toxin applied at concentrations which could be reasonably expected in or around the disease plant, produces in a susceptible host all the symptoms characteristic of the disease;

2. the pathogen and the toxin exhibit similar suscept specificity;

3. the ability of the pathogen to produce the toxin varies directly with the ability to cause disease; and

4. a single toxin is involved.

The same authors coined the term "phytotoxins" for all products of living organisms toxic to plants, without reference to their role in plant diseases. Pringle and Scheffer (1964) proposed "Host-specific toxins" to include the metabolic products of the pathogenic microorganisms which are toxic only to the host-susceptible to the pathogen. In addition to these, Wright (1968) coined the term "Mycotoxins" to include all toxins of fungal origin. The literature pertaining to the toxins produced by plant pathogenic fungi has been reviewed below. For convenience, the literature is grouped into two groups as Toxins produced by (i) Facultative parasitic fungi, and (ii) obligate parasites.

Toxins produced by facultative parasitic fungi

Helminthosporium toxins:

The toxic activity in culture filtrate of *Helminthosporium victoriae* causing blight of oats was reported by Meehan and Murphy (1946). Litzenberger (1949) detected the toxin in infected plants in sufficient concentration to bring about the effects similar to those obtained in pure culture. Wheeler and Luke (1954) obtained high yields of *H.victoriae* toxin by growing it in chemically defined medium. The toxin was partially purified and designated as "victorin". They also obtained

a mutant which was highly pathogenic and produced high yields of toxic compound. Pringle and Braun (1957) purified the toxin and noted that victorin inhibited the growth of susceptible oat roots at a concentration of 0.01 µg/ml. They found biologically active victorin to be a polypeptide in nature. The toxin production was restricted to only pathogenic isolates and selected high toxin yielding strains (Scheffer et al., 1964). The toxin production was genetically controlled and influenced by age of the cultures used (Nelson et al., 1963). Pringle and Braun (1958) degraded the purified toxin by adjusting the pH of aqueous solution with sodium bicarbonate. The degraded toxin gave a strongly positive ninhydrin reaction and when chromatographed on paper, two spots appeared with Rf values of 0.67 and 0.84. In 1960, they found out the empirical formula of the latter compound with the Rf value of 0.84 as $C_{17}H_{29}NO$ and named it as "victoxinine". This compound was found to be equally toxic to toxin-susceptible and resistant varieties indicating that it was non-host specific and it may be isolated directly from culture filtrates of poor or non-toxin producing isolates of H.victoriae. Nishimura et al., (1966) reported the appearance of victoxinine before victorin in culture medium.

Krupka (1958) and Scheffer and Pringle (1963) observed that victorin increased 3-5 fold oxygen uptake by susceptible oat tissues but not by resistant oats. Also the ascorbic acid oxidase activity was found to be 2-4 fold as great in susceptible oat homogenates but it reduced the transpiration in both resistant and susceptible oat varieties (Litzenberger, 1949 and Wheeler, 1968). Studies with respiratory

inhibitors by Krupka (1959) revealed that 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation had no effect on victorin treated susceptible oat tissue. However, sodium fluoride reduced the high respiratory level of victorin treated plants and sodium diethyldithiocarbamate and phenyl thiourea, two inhibitors of copper containing enzymes had a greater effect on victorin treated tissues. Malonic acid, a Kreb's cycle inhibitor had the reverse effect. Luke and Freeman (1962a) reported that victorin treatment caused an increase of free amino acids in susceptible varieties. The increase of free amino acids coupled with a decrease of bound amino acids indicated that toxin treatment disputes protein metabolism. While studying the effects of victorin on Kreb's cycle intermediates of susceptible oat variety, Luke and Freeman (1965) found that victorin caused increased production of malic acid through a CO_2 fixation reaction. It also enhanced citric acid synthesis but had no effect on aconitic and succinic acids. The amino acids uptake was decreased after exposure to 2.5×10^7 dilution of a toxin (Scheffer, 1964).

An increased permeability of both the plasma membrane and tonoplast of root cells in susceptible cultivars but not in resistant cultivars treated with victorin was observed (Black and Wheeler, 1962a; Wheeler and Black, 1962 and 1963; and Keck and Hodges, 1973). The Victorin treatment caused breakdown of the nuclear membrane, collapse and aggregation of cytoplasm and modification of mitochondria, disruption of the internal membrane systems, lysis of plasma membrane, chloroplast membrane, and

disorganization of grana framework system of chloroplasts (Hanchy and Wheeler, 1965; Luke et al., 1966; and Samaddar and Scheffer, 1966). The loss of the ability of root hair cells to plasmolyse, uptake of exogenous amino acids and inorganic phosphorus, incorporation of P into organic P and amino acids into proteins and protoplasmic streaming, were recorded after the victorin treatment in susceptible cultivars (Samaddar and Scheffer, 1968). Increased leakage of electrolytes and phosphorylated sugars from victorin treated tissues were recorded (Black and Wheeler, 1962b; Black, 1963; Luke et al., 1969; and Gardner et al., 1974).

The reaction of the plants to victorin indicated that the uptake of toxin by susceptible plants may be due to receptor sites present in susceptible plants to which toxin gets adsorbed as reported by Luke and Freeman (1962b) and Hanchy and Wheeler (1965). This receptor site was found to be absent in resistant varieties (Hanchy and Wheeler, 1965). The statement was disproved by Doupnik and Wheeler (1965) showing that victorin is adsorbed even by resistant variety. Samaddar (1968) suggested that the carbonyl group may be involved in binding the victorin on the membrane. Doupnik (1968) noted the suppression of victorin induced symptoms by treatment of $CaCl_2$. Hanchy (1969) reported suppression of victorin toxicity in oats, when pretreated with Uranyl salts. Gardner and Scheffer (1969, 1970 and 1973) found that when susceptible plants were treated with cycloheximide and sulfahydryl binding compounds, the victorin induced electrolyte leakage was reduced. Saftner et al. (1976)

suggested that selective effects of victorin may be caused by a victorin induced deficiency of calcium in susceptible but not in resistant tissue.

A host-specific toxic metabolite from Helminthosporium carbonum was reported by Scheffer and Ullstrup (1965). They noted the inhibitory action of culture filtrate only when susceptible corn was the test plant and had no inhibitory effects on resistant corn seedlings. Well developed seedlings of susceptible corn developed necrosis when placed in a culture filtrate but had no effect on resistant seedlings. Kuo and Scheffer (1967) and Kuo et al. (1970) while studying the comparative effects of H.carbonum and H.victoriae found that HC-toxin did not stimulate O_2 uptake in tissues and incorporation of C^{14} amino acids and uridine into trichloroacetic acid insoluble plant components was not affected. It did not cause electrolyte leakage from susceptible tissue nor the activity was affected by sulfite. Very dilute solutions of the HC-toxin stimulated root growth of corn seedlings. Pringle and Scheffer (1967b) isolated a host-specific toxin and a related substance with non-specific toxicity from H.carbonum. The non-specific toxin was referred as carbtoxinine. They crystallized HC-toxin and found that crystalline toxin did not react with ninhydrin but yielded amino acids on acid hydrolysis. Kuo and Scheffer (1968 and 1969) noted that toxin uptake by susceptible corn seedlings was affected by H.carbonum toxin concentration and the time of exposure to the toxin. The toxin uptake was temperature dependent and was decreased by 2,4-dinitrophenol, sodium azide and potassium cynide.

The toxin increased the CO_2 uptake in tissue extracts. Yoder and Scheffer (1969) noted the stimulatory effects of lower concentrations of H.carbonum toxin on corn seedlings.

Pringle (1970) found out its molecular weight which was 700 and empirical formula as $C_{32}H_{50}N_6O_{10}$. He identified the compound as cyclic peptide having alanine, proline and three other ninhydrin positive compounds. The production of amines similar to victoxinine by H.carbonum was reported by Pringle and Scheffer (1970). Pringle (1971) studied the amino acid composition of the host-specific toxin of H.carbonum.

Yoder (1970) reported that HC-toxin caused 30-70 per cent increase in NR activity and also the nitrate reduction induction was increased. Toxin did not affect NR activity in the absence of NR synthesis (without NO_3^-), nor did toxin change the rate of NR degradation. The increased adsorption of nitrate and sodium and reduced adsorption of potassium, sulphate and phosphate ions due to toxin was observed by Yoder and Scheffer (1971). Comstock and Scheffer (1970 and 1973) reported the necessity of toxin and toxin sensitive tissue for successful colonisation of H.carbonum. Yoder and Scheffer (1973a and 1973b) proved that the nitrate reduction in toxin treated susceptible tissue was not due to nitrate reductase. They explained that increase in nitrate uptake and accumulation of nitrate was due to availability of substrate but not due to increase in metabolism. They suggested that toxin action was not by

derangement of plasma membrane instead it may cause specific changes in plasma membrane, which might have increased the uptake of certain solutes. The abolishment of specific toxicity of host-specific toxin of H.carbonum by electrolytic reduction was noted by Pringle (1973). Hoffman and Zscheile (1974) detected increased peroxidase activity in toxin treated or inoculated corn tissues.

A partially purified host-specific toxin from H.sacchari causing an eye-spot disease of sugarcane which is characterized by a long chlorotic streaks free of fungus was isolated by Steiner and Byther (1969). Byther and Steiner (1971) used host-specific toxin in determining the reaction of sugarcane seedlings to eye-spot disease. They concluded that eye-spot susceptible seedlings can be eliminated from a population by spray application of toxin. The partial characterisation of H.sacchari host-specific toxin was done by Steiner and Byther (1971) and also found that plants susceptible to fungus were affected by the toxin. They suggested the use of toxin for screening of sugarcane varieties for resistance to eye-spot disease. Steiner and Strobel (1971) purified and named the compound as "Helminthosporoside". They proposed the structure of helminthosporoside as 2-hydroxy-cyclopropyl-alfa-D-galacto-pyranoside. The helminthosporoside was recovered from a runner on an infected leaf and it was purified (Strobel and Steiner, 1972). Byther and Steiner(1972) used helminthosporoside to select sugarcane seedlings resistant to eye-spot disease. The studies with ultrastructures of cells in toxin treated

and H.sacchari infected susceptible sugarcane leaves by Strobel et al. (1972) revealed virtually no alteration of the cytoplasm to its complete disruption and abnormalities in the ultrastructure of chloroplasts. Byther and Steiner (1974) gave a possible mechanism of host induced resistance to helminthosporoside. Strobel (1973a) found that susceptible hosts possessed membrane protein which can bind the toxin. He purified the binding protein and determined its molecular weight as 45,000. It was found to have 4 sub-units of molecular weight 11,700 and atleast 2 binding sites each. Further, experiments by Strobel (1973b) showed that the binding protein of resistant clones differed with that of susceptible clones in having low electrophoretic mobility and four different amino acid residues. Strobel and Hess (1974) gave the evidence for the presence of a toxin binding protein on the plasma membrane of the plant cell. The studies with the toxin binding protein of sugarcane by Strobel (1974b) revealed that it possessed raffinose binding activity, it participates in alfa-galactoside transport and activated K^{+} - Mg^{++} ATPase enzyme. Strobel et al.(1975) showed the lack of toxin binding protein activity in mutants of sugarcane clone obtained by irradiation. Steiner and Byther (1976) compared and characterised the toxin produced by H.sacchari from Australia, Florida and Hawaii. Pinkerton and Strobel (1976) obtained the production of helminthosporoside in a medium by attenuated cultures by adding the material obtained from the water wash of the susceptible sugarcane leaves. One activator was identified as serinol which activated toxin production in attenuated cultures at 1 µM.

Smedegard-Petersen and Nelson (1969) demonstrated the production of a host-specific toxin from isolates of H.maydis causing blight reactions on corn. They detected toxin by following bioassay methods viz., detached corn leaves and seedling assay. From the experiments they noted that toxin production was under genetic control. Linderberg (1971) noted that diseased isolates of H.maydis produced more toxin than the healthy isolates. The host-specific toxin of H.maydis was purified and some of its properties were studied by Comstock (1971). He observed that toxin was heat labile, activity was reduced by 50 per cent in 2-4 hr at 25 C and pH 7.0, less than 20 per cent at pH 3.5 and toxin had an approximate Rf value of 0.85 on paper chromatograms. The production of different specific toxins by different isolates of H.maydis was reported by Linderberg (1972). Wheeler et al.(1972) investigated the possibility of using mitochondrial sensitivity to the fungal toxins of H.maydis for detecting resistant plants. The permeability changes induced by H.maydis race T toxin were studied by Graeen et al. (1972). Comstock and Scheffer (1972) noted that isolates of H.maydis race T differed greatly in the ability to produce host-specific toxin and the toxin production was varied with the composition of the medium and age of the cultures. No host-selective toxin was detected from H.maydis race O.

The pathotoxin from H.maydis caused a rapid inhibition of photosynthesis, induced stomatal closure and inhibited K^+ uptake by guard cells (Arntzen et al., 1973). Arntzen et al. (1973) studied the effect

of H.maydis (race T) pathotoxin on energy linked processes of corn seedlings. Tipton et al. (1973) observed the inhibition of the K^+ stimulated ATPase of maize root microsomes by H.maydis race T pathotoxin. The reaction of germinating maize pollen to H.maydis pathotoxins was studied by Laughman and Graby (1973). Mertz and Arntzen (1973) found that the toxin depolarized the electrical potential in susceptible maize varieties.

Lim and Hooker (1972) characterized the toxins from H.maydis race T and race O. H.maydis race T toxin was having Rf value of 0.95 and found to be involved in disease development. Helminthosporium race O toxin was having Rf value 0.87 and had no role in disease development. Karr et al. (1974) isolated four host-specific toxins and found that toxins I and II were chemically similar and toxin III was a glycoside chemically similar to toxin I and II. Halloin et al. (1973) reported that the toxin is host-specific only with respect to electrolyte leakage but not with respect to leakage of carbohydrates from root and leaf tissue of corn. A simple biochemical assay for "Texas" cytoplasm in corn by the use of H.maydis race T pathotoxin was developed by Peterson et al.(1974). Pelchar et al. (1975) reported that the susceptible protoplasts exposed to toxin inhibited cytoplasmic streaming. The inhibition of electron transport in maize mitochondria by H.maydis race T pathotoxin was reported by Flavell (1975). Bhuller et al.(1975) reported the inhibition of dark CO_2 fixation and photosynthesis in leaf discs of corn susceptible to the host-specific toxin produced by H.maydis race T. The involvement of H.maydis race T

toxin during colonization of maize leaves was reported by Comstock and Martinson (1975). Karr and Hsu (1975) reported 5 chemically related host-specific toxins from H.maydis race T. Bednarski et al. (1977) reported increased O_2 uptake by toxin treated leaves and coleoptiles from susceptible plants, inhibited glycolysis and decreased levels of acid labile organic phosphates in susceptible tissues. They reported that mitochondrial site may be the only important site for direct action of the toxin.

Ludwig (1957) noted toxin production by H.sativum and its role in pathogenicity. Linderberg (1969) reported disease induced toxin production in H.oryzae culture filtrate.

Alternaria toxins:

Strains of Alternaria tenuis are known to produce many compounds like Alternariol and Alternariol monomethyl ether (Raistrick et al.,1953); altenuic acid, altenuisin, altenuol (Rosett et al., 1957); altenuridi (Pero et al., 1973) and Senazoic acid (Rosett et al., 1957; and Stickings, 1959). None of these compounds were isolated for toxin studies.

The toxic principle isolated from culture filtrates of A.tenuis caused chlorosis when applied to cotyledonary leaves of the seedlings of cotton, citrus, and cucumber (Fulton et al., 1960). Templeton et al. (1965 and 1967) studied the factors affecting the induction of chlorosis

due to A.tenuis toxin. Fulton et al. (1965) isolated a metabolite from A.tenuis which inhibited chlorophyll production in the seedlings of okra, pea, bean, cucumber and watermelon. Similar metabolite was produced by A.citri and A.longipes. Grable et al. (1966) and Templeton et al. (1967) purified the toxin from A.tenuis which was peptide based. They gave the molecular formula of A.tenuis toxin as $C_{24}H_{32}N_4O_4$ and named as "Tentoxin". Saad et al. (1969 and 1970) purified the tentoxin and standardised a bioassay method for its activity. Halloin et al.(1969 and 1970) studied the effects of tentoxin on chlorophyll synthesis and plastid structure in cucumber and cabbage. They noted that the toxin acts through disruption of the normal plastid development rather than through direct interference with chlorophyll synthesis as reported by Fulton et al. (1965). Durbin and Uchytil (1972) reported that tentoxin affect the transpiration and opening of stomata. Asaldin and Patil (1972) isolated a phytotoxic substance produced by A.tenuis affecting passion fruit vines in Hawaii. Arntzen (1972) suggested that the tentoxin is an energy transfer inhibitor which acts at the terminal step of ATP synthesis. Durbin et al. (1973) reported that tentoxin induced closure of stomata, retarded stomatal opening and the uptake of potassium by guard cells was inhibited. Woodhead et al.(1975) gave procedure for crystallisation and further purification of tentoxin.

The selective toxicity of Alternaria kikuchiana was reported by Tanaka (1933) and toxin was isolated by Hiroe et al.(1958). Otani et al. (1975) studied the role of host-specific toxin in early step of infection

and noted increased loss of electrolytes from susceptible leaves after toxin treatment. Otani et al. (1976) reported that exposure of tissues to abscisic acid decreased the sensitivity and indole-3-acetic acid on the other hand, gave a partial promotion. Park et al. (1976) studied the effects of the host-specific toxin from A.kikuchiana on the ultrastructure of plasma membranes of cells in leaves of Japanese pear. Park (1977a) found the origin of inclusive materials between cell walls and invaginated plasma membranes. Park (1977b) studied the effects of the host-specific toxin and other toxic metabolites produced by A.kikuchiana. Park (1977c) studied ultrastructural changes in cells of susceptible leaves of Japanese pear. Morikawa et al. (1977) studied the efflux of cell constituents from pear leaves treated with Ak-toxin.

Pound and Stahman (1951) reported the production of a toxic substance by A.solani and its relation to the early blight of tomato. Ueno et al. (1975 and 1975) isolated and characterized the host-specific AM-toxin I and II produced by A.mali causing blotch of apple. Padmanabhan and Narayanswamy (1975) reported the toxin from A.macrospora a causal agent of leaf spot. A host-specific toxin produced by A.alternata f. sp. lycopersici was reported by Gilchrist and Orogan (1976). Maity and Samaddar (1977) reported a toxic metabolite from A.eichhorniae causing blight of water hyacinth and studied its properties. Toxins produced by species of Alternaria has been excellently reviewed by Templeton (1972).

Periconia circinata toxin:

The culture filtrate of Periconia circinata, causal agent of milo disease of sorghum, inhibited the root growth of susceptible variety at dilutions of 1:3200 (Scheffer and Pringle, 1961). Pringle and Scheffer (1963) purified the toxin and found that it was polypeptide in nature. Pringle and Scheffer (1966) crystallized the toxin and its further analysis revealed that toxin to be a polypeptide of alanine, aspartic acid, glutamic acid and serine in the ratio of 6:4:2:2. The production of two different host-specific toxins which were named as P.circinata toxins A and B by Periconia circinata was reported by Pringle and Scheffer (1967a). Gardner et al. (1972) reported that P.circinata toxin increased loss of electrolytes with increased toxin concentration. The toxin decreased the ability of tissue to take up and/or to retain amino acid incorporation into insoluble components of the cell. The cycloheximides, phospholipase and uranyl salts decreased the sensitivity of tissues to toxin.

Phyllosticta maydis toxin:

Comstock et al. (1972) and Yoder (1973) reported the production and studied the characteristics of a host-specific toxin from Phyllosticta maydis causing yellow blight of corn leaves. Comstock et al. (1973) reported that the toxin selectively inhibited seedling root growth, induced leaf chlorosis and caused an increased leakage of electrolytes from maize leaves containing Texas male sterile cytoplasm. The toxin treatment of mitochondria caused an immediate irreversible swelling, uncoupled oxidative phosphorylation and (depending upon substrate) either

stimulated or inhibited O_2 uptake. Comstock and Martinson (1974) chromatographically compared toxins of Phyllosticta maydis and Helminthosporium maydis and did not find any difference in their Rf values and elution volume suggesting their probable similar nature.

Fusarial wilt toxins:

As early as in 1923 a toxic excretory product of Fusarium solani capable of wilting tomato cuttings was reported by Fahmy. Gottlieb (1943) detected the presence of toxins in tomato plants affected with fusarial wilt. The virulent strains of Fusarium produced more toxic compounds compared to mild strains (Wellman, 1943). A toxic polysaccharide from F.solani f. eumartii was reported by Thomas (1949). Kalyansundaram and Venkatram (1956) detected fusaric acid in culture filtrates of Fusarium vasinfectum causal agent of cotton wilt. Gaumann (1957) reported the production of fusaric acid by other members of family Hypocreaceae in addition to Fusarium vasinfectum and F.lycopersici. He also explained that fusarial wilt symptoms are due to 5 other compounds apart from fusaric acid. Some of the compounds are fusaric acid, dehydrofusaric acid, alfa picolinic acid, phytotonievein, novorubin, lycomarasmin, etc., according to Owens (1969). Gaumann (1958) reported the mechanism of fusaric acid injury. Page (1959) found the fusaric acid in banana plants infected with Fusarium oxysporum f. cubens. Fusaric acid has been detected in culture filtrates of many species of fungi (Kalyansundaram and Venkatram, 1956; Gaumann, 1957; Collins and Scheffer, 1958; Kuo and Scheffer, 1964; and Davis, 1969). Chemically

fusaric acid is a pyridine carboxylic acid (Gaumann, 1957) found to increase respiration and mitochondrial activity at low concentrations. At higher concentrations caused a drastic decrease in succino oxidase activity in mitochondria as reported by Kuo and Scheffer (1964). Many other compounds produced by Fusarium sp. have been reviewed by Dimond (1955), Wright (1968), Owens (1969) and Strobel (1974). The production of toxic substances particularly trichothecene derivatives by Fusarium oxysporum F. sp. carthami was reported by Ghosal et al. (1976). Lakshminarasimhan and Kalyansundaram (1977) correlated the virulence of three Indian and two American isolates of Fusarium vasinfectum with their aggressiveness - the capacity to colonize the host vasculature. They found that on their non-congenial hosts, the isolates were circumscribed to the host cortex. They observed Indian isolates synthesized the toxin, fusaric acid, in vitro on 'living roots' of the congenial host G.arboreum, but the American isolates failed to do so. None of the Indian isolates could produce fusaric acid when grown on 'living roots' of G.hirsutum. Malathi and Kalyansundaram (1977) studied the changes in the host-constituents like cations and anions in the xylem of G.arboreum and G.hirsutum plants infected by Indian and American strains of Fusarium vasinfectum. They noted differences of the two host species to their respective anions in the xylem sap of G.arboreum and G.hirsutum. They observed a decrease in the levels of the most of the constituents studied.

Fusicoccin amygdali toxins:

A purified toxic compound from culture filtrates of Fusicoccin amygdali causing wilting of oak was reported by Ballio et al. (1964). The major active compound amongst seven toxic compounds was purified and named as "Fusicoccin". It's non-specific nature and reversible wilting effect were reported by Chain et al. (1971). Ballio et al. (1968) gave its structure. Besides Fusicoccin, Ballio et al. (1970) identified six more compounds which they designated as F II to F VIII which were later identified by Ballio et al. (1970 and 1972). Turner and Granity (1969) found it to be a tricarboxylic terpene with a molecular weight of 680. Turner and Granity (1969) and Squire and Mansfield (1974) reported that the toxin stimulated stomatal opening thereby increasing permeability of cells to potassium ions. Squire and Mansfield (1974) evidenced the ability of the Fusicoccin to overcome the environmental factors which inhibit the stomatal opening. Lado et al. (1972 and 1974) reported that Fusicoccin was more active than indole-3-acetic acid, gibberlic acid and benzyl adenine.

Miscellaneous fungal toxins:

Barnum (1924) reported the production of substances toxic to plants by Penicillium expansum a saprophyte. The production of toxin by Ceratostomella ulmi causing Dutch elm disease was reported by Zentmyer (1942). The physiology of toxin production by the same fungus was

studied by Feldman *et al.* (1950). Porter and Green (1952) reported production of exotoxin in the genus *Verticillium*. White and Fredrick (1954) reported toxin production by oak wilt fungus *Ectoconidiophora fagacearum*. Goodman (1960) reported colletotin, a toxin produced by *Colletotrichum fuscum*. Sally and Goodman (1962) studied the morphological effects of colletotin on tomato and *Digitalis* foliage. Sherwood and Lindberg (1962) reported the production of a phytotoxin by *Rhizoctonia solani*. Miller (1966) noted production of toxin by *Dothidella ulei in vitro*. Selemink *et al.* (1966) purified the phytotoxic compound from culture filtrates of *Didymella applanta*. Bassett *et al.* (1967) reported production and biological activity of formannosin, a toxic sesquiterpene metabolite of *Fomes annosus*. The toxin produced by *Ceratocystis fagacearum* was partially purified (Gergor, 1969) and its general characteristics were studied by Gregory and McWain (1969). Cunfer and Lukezic (1970) studied the possible role of toxin from *Myrothecium roridum* in leaf spot of red clover. Balis and Payne (1971) reported a toxin from *Cercospora beticola*. It was toxic to sugarbeat leaves. It was found to contain cercosporin and mixtures of triglycerides and some autolysed products of fatty acids. The formula of cercosporin was suggested as $C_{29}H_{26}O_{10}$. A toxic factor from *Pyrenochaeta terrestris* was reported by Hess *et al.* (1972). A non-host specific toxin producing necrotic spots on sunflower leaves was isolated from broth cultures of *Sclerotium bataticola* (Chan and Sackton, 1973). Dhingra and Sinclair (1974) isolated a phytotoxin from culture filtrates of *Microphomina phaseolina* (*Rhizoctonia bataticola*) and from the leaves infected by the same organism.

Bousquet and Skejennikoff (1974) isolated and purified an active compound from culture filtrates of Septoria nodorum, causal agent of glume blotch of wheat. Gray and Chamberlain (1974) gave an evidence for toxin production by Cephalosporium gregatium. Issac et al. (1975) isolated and characterized stemphylin, a chromone glucoside from Stemphylium botryosum. Toxin produced by Pyricularia oryzae was reported by Rao et al. (1974) and Reddy and Prasad (1975).

Balakrishna (1975) isolated a toxic but non-specific glycopeptide from culture filtrates of Septoria lycopersici a causal agent of leaf spot disease of tomato. He isolated the toxin from the plants infected with S.lycopersici. The toxin contained two fractions having molecular weight 129,200 and 9078, the fraction II was more toxic. He found that toxin was antigenic but only fraction II was serologically related to crude toxin. Kent and Strobel (1976) reported a phytotoxin from Septoria nodurum a causal agent of glume blotch of wheat. The toxin was non-specific, unstable and a low molecular weight cationic acid. Smedegard-Peterson (1977a) isolated two toxins produced by Pyrenophora teres and studied their role in disease development of net-spot blotch of barley. The respiratory changes of barley leaves infected with Pyrenophora teres and affected by isolated toxins of this fungus were studied by Smedegard-Peterson (1977b).

Toxins produced by obligate plant parasites

Litzenberger (1949) obtained a toxic extract from resistant Victoria oat plants infected with crown rust, the toxin caused a wilting

and a depression in developing seedlings of Vicland (a derivative of Victoria oats) as evidenced by reduced root and top growth. The toxin was obtained from resistant hosts but not from susceptible hosts and was specific for Victoria oats and its derivatives.

Swaebly (1960) demonstrated that germinating urediospores of race 15B of Puccinia graminis var. tritici produced a substance that, introduced into Kentana Wheat seedlings under reduced pressure, caused a necrotic reaction similar to that occurred with natural infection. Olien (1956) using electrophoretic treatments during certain stages of pustule development, demonstrated that the necrotic area resulting from the infection of Khapli emmer with race-56 of wheat stem rust could be displaced from the region of mycelial invasion towards the anode. He postulated that a negatively charged toxin produced by the disease complex causes the necrotic response of Khapli emmer to infection by the race of wheat stem rust used.

Silverman (1960) extracted a host-specific toxin from Puccinia graminis var. tritici race-38 from infected Marquis wheat. He noted that toxin which caused chlorosis in test Marquis wheat seedlings was extractable from infected plants grown at a higher temperature (90°F) but not from those grown at a lower temperature (70° F). Chlorosis appeared in seedlings of Marquis infiltrated with the toxin only when these test plants were grown at a low temperature but did not appear on plants

grown at high temperature. Little Club wheat, which was susceptible to race-38 at all temperatures did not become chlorotic following infiltration with the toxin regardless of the temperature, preceding or following infiltration. The toxin causing chlorosis was a water soluble, heat-stable compound that retained its toxicity for atleast four months at -10° C.

Rai (1977b) isolated a toxic compound produced by Plasmopara viticola infected leaves and further purified and characterized. The toxic compound caused wilting of tomato plant cuttings and also caused a chlorotic spot on the grape leaves on spot tests. The molecular weight of toxic compound was 89,760. It was composed of galacturonic acid, gluconic acid, glucoronic acid, mannuronic acid and three unknown organic acids. The cationic fraction composed of arginine, aspartic acid, glutamic acid and glycine. The neutral fraction yielded galactose, glucose, mannose, melibiose, raffinose and xylose. The toxin was non-specific and a glycopeptide in nature.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

Inoculation of bajra plants with Sclerospora graminicola

For obtaining the bajra plants infected by Sclerospora graminicola causing green ear or downy mildew disease, seeds of H.B.-3 variety which is most susceptible to this fungus were sown in 10" diameter plastic pots under greenhouse condition. Young bajra plants of 6-7 cm height were inoculated with the sporangia of S.graminicola as described below.

Bajra leaves infected by S.graminicola were collected in the evening from the Main Research Station farm of the University of Agricultural Sciences, Bangalore. The downy growth on the infected leaves, if any, was washed off with gentle flow of water in order to remove the sporangiophores produced during the previous night. The leaves were cut into 10 cm length pieces and kept in moist chamber with the ventral surface of the leaf in contact with moist filter paper. After incubating for 8-10 hr a good crop of sporangiospores was obtained under laboratory conditions. The sporangia were harvested in distilled water rubbing off the downy growth by using camel hair brush. The young HB-3 bajra seedlings were inoculated between 5.30 and 6.00 a.m. by putting sporangial suspension thus prepared in the whorl of the opening leaf with the help of hypodermic syringe. The inoculated plants were kept

in shade and covered with polythene bags to avoid direct exposure to the sun and evaporation of sporangial suspension from the leaf whorl. Corresponding control plants of bajra inoculated with only distilled water were maintained.

Maintenance of culture

The soil in the cement pots of size 18" x 12" x 12" was made downy mildew sick by burying infected bajra plants where the oospores were produced. In such sick soil seeds of HB-3 bajra variety were sown and the infected plants were obtained under green house conditions. In this way, throughout the year, culture of S.graminicola was maintained on bajra plants.

Extraction and partial purification of toxin from infected bajra plants

The infected plants showing typical downy mildew symptoms viz., chlorosis, downy growth, wilting or green earhead were selected. The selected plants were air dried and powdered in mechanical grinder. About 200 g powder of infected bajra plants was soaked in 100 ml distilled water for 2-3 hr and then homogenized with 500 ml distilled water in Sorvall Omni mixer. The extract was filtered through four layers of cheese cloth, filtered through Whatman No.42 filter paper and then centrifuged at 15,000 rpm for 15 min in refrigerated Sorvall centrifuge. The supernatant was collected and the pellet was discarded. The clear supernatant was concentrated to 1/4th of its volume by evaporating

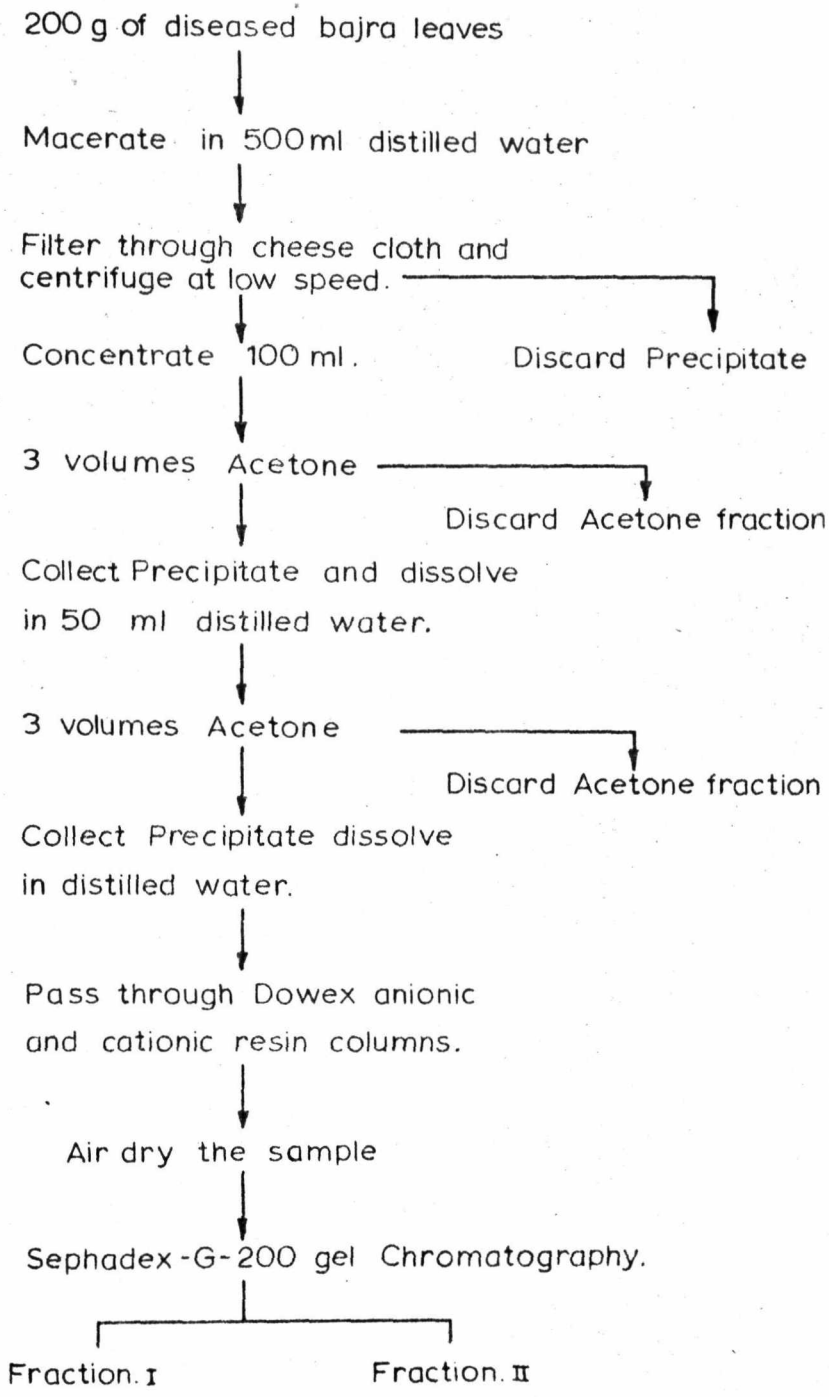


Fig. 1 Flow sheet summarizing the procedure used to purify Sclerospora graminicola toxin.

at 40° C by vacuum flash evaporation. The concentrated supernatant was precipitated by adding 2-3 volumes acetone with constant stirring and allowed to stand at 4 C overnight. The supernatant was slowly decanted out without disturbing the pellet settled at the bottom of the beaker. The pellet was collected by centrifuging the remaining material at 10,000 rpm for 10 min. The pellet was collected in beaker and dried completely by using compressed air and stored in vacuum desiccator over P_2O_5 and NaOH. The dried compound was dissolved in 10 ml distilled water and then passed through charged 10.0 x 2.0 cm columns of anionic Dowex-1 (200-400 mesh formate form) and cationic Dowex-50 (200-400 mesh, H^+) resins. The effluent was collected, air dried and stored in vacuum desiccator containing P_2O_5 and NaOH; such a preparation was termed as crude toxin. By following the same procedure healthy bajra plants were also extracted for comparison. The flow sheet summarizing the procedure used to extract and purify S.graminicola toxin is given in Fig.1. The fractions obtained after passing the Sg-toxin through Sephadex Gel column were referred as Fraction I and II respectively (Fig.6).

Bioassay of crude toxin

Two-fold dilutions starting from 2.0 per cent level were prepared by dissolving compounds obtained from healthy and diseased bajra plants in distilled water. Young 10 days old plant cuttings of HB-3 bajra variety were put in serially diluted samples. Tomato plant cuttings have been used for testing toxicity of several microbial polysaccharides, other polysaccharides and other large molecules (Hodgson et al., 1947 and 1949). Tomato cuttings were also tried for testing toxicity.

Comparison of toxicity of crude toxin to fractions I and II

Two-fold serial dilutions starting from 2.0 per cent level were prepared by dissolving crude toxin, and fractions I and II in distilled water. Tomato plant cuttings were put in the diluted compounds. The time required to show symptoms was recorded in each case. The proper control of healthy plant extract was maintained. During this experiment, plants were exposed to artificial illumination and aeration.

Biological properties of the toxin

The crude toxin was used to study the biological properties by testing the seed germination of toxin treated seeds, detecting its anti-microbial property and observing its effect on plant growth as described below:

Effect of toxin on seed germination, radicle and plumule lengths

This experiment was conducted by using seeds of eight different crop varieties belonging to the families Poaceae, Fabaceae and Solanaceae. Seeds were selected and surface sterilized by treating them in 0.1 per cent mercuric chloride for two minutes and then washed thoroughly 6-7 times with sterile water. These surface sterilized seeds were soaked in different dilutions viz., 2.0 per cent, 1.0 per cent and 0.5 per cent of toxin for 12 hr. Similarly, seeds were soaked in sterile distilled water to serve as control. The soaked seeds were placed on sterile moist filter paper discs in sterilized petriplates. The plates were incubated at room temperature for 3-4 days and sterile water was added

to keep the filter paper moist as and when required. The observations of germinating seeds particularly germination percentage, radicle and plumule lengths were recorded.

Host-specificity of the toxin

Two-fold serial dilutions of crude toxin in aqueous solution starting from 2.0 per cent level were prepared and 0.5 ml of each of these dilutions was transferred into small glass vials. Ten different crop plants representing different families were tested by treating 4-5 cm plant cuttings in different test dilutions of the toxin. These test plants were exposed to artificial illumination and aeration so as to enhance uptake of the toxin. Observations on the nature of symptoms that developed and time taken for development of such symptoms was recorded.

Effect of toxin on the growth of microorganisms

To study the effect of toxin on the growth of microorganisms 23 different microbial cultures representing different groups viz., actinomycetes, algae, bacteria and fungi were used. For actinomycetes and bacterial cultures standard filter paper disc method was followed. The discs of Whatman No.1 filter paper (6 mm) were soaked in different dilutions viz., 1 per cent, 2 per cent and 4 per cent of toxin in distilled water and were put in the centre of the plate on agar surface seeded with particular culture. For fungal and algal cultures antibiotic cup assay

method was used. The sterilized steel cups were kept at the centre of the sterilized plates and then slowly seeded agar was poured. After solidifying the agar, 0.1 ml solution of different test dilutions of toxin was put in each cup. These plates were incubated for 12 hr at 4 C for allowing the toxin to diffuse in the surrounding agar medium. Then, the plates were incubated for 3 days at room temperature (30 C). Observations on presence or absence of an inhibition zone and its diameter, if present, was recorded.

Effect on plants grown from seeds soaked in toxin solution

Bajra seeds were soaked in crude toxin solution (2.0 per cent, 1 per cent and 0.5 per cent) for about 12 hr. Seeds soaked in water served as control. These soaked seeds were sown in small pots and kept at room temperature. These plants were grown for a period of 6 weeks and observed regularly for symptoms like germination, dwarfening, chlorosis and wilting of plants.

Histochemical changes in bajra seeds due to toxin

Sound, healthy bajra seeds were selected and surface sterilized and washed thoroughly in sterile water. They were then soaked in 2.0 per cent toxin solution for 12 hr. Control seeds were soaked in sterile water. The soaked seeds were germinated in petriplates containing moist filter papers and incubated at room temperature for 24 hr. These germinated seeds as well as the leaves from plants showing

symptoms (plants grown from seeds soaked in toxin) and from healthy plants were processed as follows. The leaves showing the symptoms were divided into three stages as (1) partially chlorotic, (2) completely yellow, and (3) curled and partially dried leaves.

Fixation and dehydration

The germinated bajra seeds were killed and fixed in Carnoy's B Fixative (6 parts alcohol + 3 parts chloroform + 1 part acetic acid). They were later washed in 80 per cent alcohol for 15 min and subjected to dehydration by using absolute alcohol-butanol grades at 3:1 and 1:1 proportions and treated with pure butanol twice.

Tissue infiltration and embedding

The materials were transferred from the medium of pure butanol, to small vials and the chips of paraffin were added successively until the medium reached a saturation point at the room temperature and later under the table lamp (40 watts). Finally, the materials were given changes with the molten pure paraffin in the oven at 60 C; thus replacing the last traces of butanol with paraffin. The materials were then embedded in paraffin employing paperboat method.

Microtoming

Serial microtome sections of 8 micron thickness were obtained.

Affixing the sections to slides

One per cent gelatin with a little of potassium dichromate was used as an adhesive. Xylol was used to deparaffinise the sections. After a few minutes the slides were passed through the solution of xylol and butanol (1:1), pure butanol and then absolute alcohol successively. The sections were hydrated passing through alcohol (downgrade) series.

Histochemical staining

Hydrated sections were subjected to the histochemical staining, then dehydrated and mounted in Canada balsam. Histochemical assessment was made for insoluble polysaccharides, nucleic acids and proteins. Following are the details of the histochemical procedures which are described under each substance and adopted in the present investigation.

Metabolite	Tests (From Jensen, 1962)	Indication
Insoluble polysaccharides	Periodic Acid-Schiff's (PAS) test (Hotchkiss, 1948)	Magenta colour
Starch	Iodine-potassium iodide (IKI) test (Johansen, 1940)	Brownish violet or deep blue
Proteins	Mercuric bromophenol blue method (Mazia et al., 1953)	Deep blue
Nucleic acids	Azur B method (Flax and Himes, 1952)	Deoxyribonucleic acid (DNA)-greenish Ribonucleic acid (RNA)-purple or deep blue
	Methyl green-pyronin (MGP)	DNA - green or deep blue RNA - deep purple

Serology

Preparation of antiserum

The antiserum was prepared by following the procedure given by Rai and Strobel (1968). Two rabbits were used for immunisation and the normal serum was collected from both the rabbits 15 days before immunisation. The serum was stored in small screw cap vials kept at -10°C after adding 3-4 drops of 0.1 per cent sodium azide.

One ml of 1.0 per cent crude toxin solution prepared in physiological saline (0.85 per cent NaCl) was emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly. Similarly, second injection of the same dose of toxin was given intramuscularly 15 days after the first injection. Third injection of 1 ml of 1.5 per cent toxin emulsified with an equal volume of adjuvant was given intravenously after a gap of 15 days after second injection. Fifteen days after the last injection the blood was collected by [illegible]puncturing the heart. The serum was collected from the clotted blood and centrifuged at 4000 rpm for 10 min. The pooled antiserum was stored in small screw cap vials at -10°C after adding 3-4 drops of 0.1 per cent sodium azide solution.

Precipitin test

Two-fold dilutions of the antiserum starting from 1:5 were prepared in physiological saline (0.85 per cent) and 0.2 ml of each dilution was

transferred to different serological tubes. To each of these tubes 0.2 ml of 1.0 per cent crude toxin solution was added and incubated at 37° C.

Immuno-diffusion

The procedure of Ochterlony (1958) and Hamilton (1961) was followed. One per cent agar was prepared by dissolving Difco Bacto purified agar in 0.85 per cent saline, 0.01M phosphate buffer (pH 7.00) and distilled water. The sterilised agar after adding 4-5 drops of 0.1 per cent sodium azide solution was poured in plates. The wells were dug out by using 9 mm template. The bottoms of the wells were sealed with molten agar. The antiserum was added into the central well and 1 per cent solutions of crude toxin, Fractions I and II prepared in 0.85 per cent saline were added to peripheral wells. Similarly, three sets of each type of agar plates were prepared. One set of each type of agar plate was incubated at 4°C, room temperature (23 ± 2° C) and another set at 37°C. All the plates were observed after 48 hr of incubation at particular temperature. The normal serum and 0.85 per cent saline were used as controls.

Inactivation of the toxin

Effect of storage

The dried crude toxin was stored in small screw cap vial and small beaker. The vial was kept in refrigerator and beaker was stored in

desiccator containing calcium chloride for 18 months. At monthly interval a sample was removed from both the sets and 1 per cent aqueous solution was prepared. The activity of the toxin samples was tested by using young tomato plant cuttings as described in earlier experiments for testing the toxicity of the compound.

Effect of temperature on the toxin

One per cent aqueous solution of toxin prepared and 0.5 ml solution was distributed in each small glass test tube. The test tubes containing toxin solution were maintained at various temperatures ranging from 50°C to 97° C for 10 min in thermostat water bath. After holding the tubes for 10 min at particular temperature, the tubes were transferred to icebath. The activity of the toxin was tested by using tomato plant bioassay method. The proper control i.e., without exposing the toxin solution to any temperature was maintained. The experiment was conducted in duplicate.

Effect of partial acid hydrolysis on toxin

An aqueous solution (0.5 per cent) of crude toxin was prepared in 1 $\underline{N}$ H_2SO_4. At an interval of 15, 20, 30, 60, and 90 minutes, 1 ml of acidic toxic solution was removed and neutralized by adding excess of $BaCO_3$. The pellet was discarded by centrifuging and filtering through Whatman No.1 filter paper. The filtrate was dried and kept in vacuum desiccator containing P_2O_5, NaOH for 12 hr. From the dried neutralized

compound 0.5 per cent aqueous solution was prepared and its toxicity was tested by following tomato plant bioassay method. The time required for causing wilting of plant cuttings was recorded. The proper control i.e., without exposing the toxin to 1 N H_2SO_4 was maintained.

Biochemical and physical properties of the toxin

Column chromatography

The crude toxin was further purified by passing through a column of Sephadex G-200 and eluting with distilled water. Fifteen milligrams of crude toxin was dissolved in 2 ml distilled water and placed on top surface of Sephadex column. The effluent was collected in 3 ml fractions using rotary automatic fraction collector. The absorption of these fractions was read at 540 mμ wave length by using Spectronic-20 to detect the compounds. The recovery of purified fractions from the crude toxin was calculated.

Molecular weight estimation

Column chromatography with Sephadex has been used effectively for molecular weight estimation of polysaccharides and toxins (Granath, 1965; Rai, 1977b and Balakrishna and Rai, 1978). Using Sephadex G-200, the equation for molecular weight estimation is:

$$\frac{Ve}{Vt} = 3.20 \text{ to } 0.58 \log \text{ molecular weight}$$

Where, Ve = The volume at which the sample is eluted from the column - the void volume of the column.

Vt = Total bed volume of the column which was 32 ml.

(The void volume was determined by the use of blue dextran and was 10.5 ml).

Let the elution volume be 'X' ml. Then the molecular weight can be determined by the above formula,

$$\frac{(X-10.5)}{32} = 3.20 \text{ to } \log 0.58 \text{ molecular weight}$$

$$3.20 - \frac{(X-10.5)}{32} = 0.58 \log \text{ molecular weight}$$

$$\text{Molecular weight} = \text{Antilog of } \frac{3.20}{0.58} - \frac{(X-10.5)}{(32 \times 0.58)}$$

Specific viscosity determination

The specific viscosity of the crude toxin and its purified fractions was determined by using the method similar to that of Flory (1953) and Rai and Strobel (1969). The intrinsic viscosity was calculated from specific viscosity. For determining the specific viscosity one per cent solution of crude or purified toxins was used.

pH determination

pH of the one per cent solutions of crude toxin and purified fractions I and II were determined by using digital pH meter.

Electrophoretic studies

One per cent aqueous solution (0.1 ml) of the crude toxin was applied on a 20 cm x 5 cm strip of Whatman No.1 filter paper in 1 cm streak and gently wetted with 0.01 M phosphate buffer of pH 7.00. Electrophoresis was carried out at 300 volts for one hour with the ends of the paper strip immersed in phosphate buffer containing the electrodes. After drying, half of the paper was treated with the reagent of Trevelyan et al. (1950) for reducing groups and the other half was treated with 0.3 per cent ninhydrin solution in alcohol for amino groups.

Acid hydrolysis

The crude toxin and its purified fractions were acid hydrolysed by following the procedure of Strobel (1967). The toxin was acid hydrolysed by refluxing (0.4 per cent) in 1 N H_2SO_4 at 100°C for 12 hr after which the acid was neutralized with an excess of $BaCO_3$ till the effervescence ceased. The precipitate was removed by centrifugation and filtering through Whatman No.42 filter paper.

Ion-exchange chromatography

The neutralised hydrolysate was passed through charged Dowex-1 (formate form) and Dowex-50 (H^+ form) columns to get neutral fraction (Strobel, 1967). An anionic and the cationic fractions were collected by eluting the Dowex-1 and Dowex-50 columns with 6 N formic acid and 6 N hydrochloric acid respectively, followed by elution with about 5 ml

distilled water. The different fractions were dried using a stream of compressed air and then kept in vacuum desiccator having P_2O_5 overnight. The weight of each fraction was recorded and percentage constituted by each fraction of crude and purified fractions was calculated.

Sugar analysis by paper chromatography

The neutral fractions of the crude toxin and its purified fractions I and II were spotted on Whatman No.1 filter paper along with the standards. The chromatograms were developed by using the following solvent systems:

1. n-butanol : acetic acid : water (4:1:5 v/v).
2. n-butanol : ethanol : water (5:1:4 v/v).
3. Ethyl acetate : Pyridine : water (8:2:1 v/v).

However, better results were obtained by using n-butanol : acetic acid : water (4:1:5 v/v). After drying the chromatograms the compounds were detected by treating with the reagents of Trevelyan et al. (1950). The compounds were identified by comparing with the standards and the Rg values (with reference to glucose) for unidentified compounds were calculated.

Quantitative estimation of sugars

For estimating known sugars from crude toxin and its purified fractions, the solutions of known concentrations were prepared by dissolving weighed neutral fractions from each toxin. The known volume

was spotted on Whatman No.1 filter paper in duplicate. On one paper standard sugars were also spotted and chromatograms were developed in butanol : acetic acid : water (4:1:5 v/v). The chromatogram with standard sugars was treated with Trevelyan reagents. The corresponding spots of known sugars from untreated chromatograms were cut and eluted with distilled water. The quantification of each sugar was done separately by following Nelson's method (1944) and by comparing with the standard curve of respective sugars.

Sugar acid analysis

The anionic fractions of the crude toxin and fractions I and II were spotted on the Whatman No.1 filter paper along with the standard. The chromatograms were developed in different solvent systems mentioned under sugar analysis. The chromatograms were dried and treated with the reagents of Trevelyan et al. (1950). The compounds were identified by comparing with the standards and in case of the unidentified compounds the Rg values (with reference to galacturonic acid) were calculated.

Amino acid analysis

The crude toxin and its purified fractions were analysed for their amino acid contents by using automatic amino acid analyser Model KLA-3B Hitachi Ltd., (courtesy of the Department of Biochemistry, University of Agricultural Sciences, Bangalore). The samples were

hydrolysed in 6 N HCl for 24 hr at 100°C and then the acid was evaporated and dried by using compressed air. The samples were dissolved in 2.2 pH acetate buffer and used for further analysis.

Elemental analysis

The carbon (C) and hydrogen (H) contents of the crude toxin and its purified fractions were done by following the standard procedure (courtesy of the Department of Organic Chemistry, Indian Institute of Science, Bangalore). The nitrogen (N) content was estimated by following the microKjeldahl method (Jackson, 1973). The oxygen content was calculated by deducting C, H and N contents. The empirical formula was calculated by using this data on the basis of one atom of nitrogen.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Inoculation of bajra plants with Sclerospora graminicola

Bajra plants, (6-7 cm tall, 10 days old) of HB-3 variety were inoculated with sporangiospores of Sclerospora graminicola. The sporangial inoculation gave more and early infection than the oospore inoculation method under green house condition. In the plants inoculated with sporangiospores, initial symptoms of chlorosis of leaves appeared within 4-5 days and later developed yellowing. Further, the infected plants showed stunted growth and wilting symptoms in about two weeks. When very young plants (3-4 cm tall, 4-5 days old) were inoculated the first symptom that appeared was wilting without showing chlorosis. If the inoculated plants survived, they developed typical green earheads. When bajra plants were grown on sick soil 100 per cent infection was observed, but slower in comparison to the sporangial inoculation. In control plants, inoculated with only distilled water no incidence of downy mildew was observed.

Extraction and partial purification of the toxin

The toxin was isolated from the powdered, downy mildew infested bajra plants by the method summarised in Fig.1. This toxin was filtered through Whatman No.42 filter paper and then through appropriate Dowex

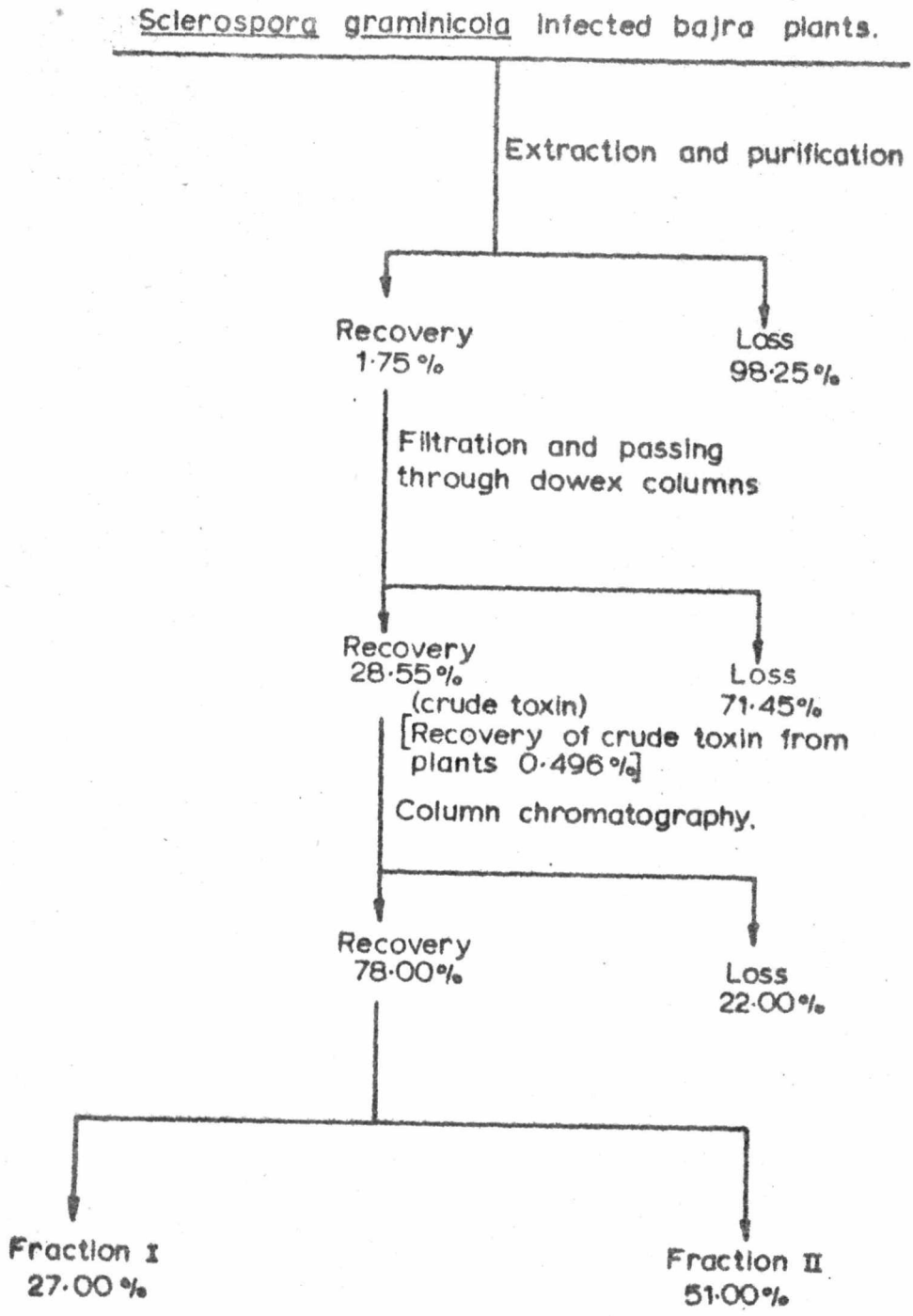


Fig. 2 Flow sheet summarizing the percentage recovery of Sclerospora graminicola toxin during its extraction and purification processes.

ion exchange resin columns. Such preparation was referred as Sg-crude toxin. Similarly, extracts from healthy bajra plants of the same age were taken.

The recovery of Sg-crude toxin was estimated as shown in Fig.2. It was observed that during the first step of extraction about 98.25 per cent material was lost as solid waste while passing through cheese cloth and then precipitation by acetone. The precipitated compound constituted only 1.75 per cent of the total plant material on dry weight basis. During the second step of partial purification i.e., passing through filter paper and then through Dowex resin columns only 28.55 per cent of the total precipitated compound was recovered and remaining about 71.45 per cent compound was lost. From these data, it was calculated that the recovery of Sg-crude toxin from infected bajra plants was only 0.496 per cent and 99.51 per cent plant material was lost during first two steps of Sg-toxin purification. These results indicated that Sg-crude toxin was present in infected bajra plants in a very low quantity.

Bioassay of crude toxin

The toxicity of the compounds extracted from healthy and downy mildew infected bajra plants was tested by using bajra plant cuttings and tomato plant cuttings. Two-fold serial dilutions of the isolated compounds starting from 2.00 per cent were prepared in distilled water and plant cuttings were put in these test solutions.

Plate 1

Bioassay of Sg-crude toxin by using tomato plant cuttings.

C = Tomato cuttings treated in 2.0 per cent solution of compound isolated from healthy bajra plants.

1) Tomato cuttings treated in 2.0 per cent Sg-toxin solution.

2) 1.0 per cent Sg-toxin solution.

3) 0.5 per cent Sg-toxin solution.

4) 0.25 per cent Sg-toxin solution.

Plate: 1

The preliminary studies showed that tomato plants expressed wilting symptoms earlier as compared to bajra plants. So, for further studies tomato plant cuttings were used to test the toxicity. The highest dilution which proved to be toxic to tomato plant cuttings was 0.06 per cent and the time required for wilting was 210 min. The time required to cause symptoms at various concentrations of Sg-crude toxin is given in Table 1 and Fig.3. The compound extracted from the healthy bajra plants did not cause wilting or any type of symptoms even at 2.00 per cent level. At the beginning, leaves of the plants in toxin solution lost the turgidity and then curling of leaves from tip towards petiole started. The plants started drooping down and showed wilting symptoms. The stems of the wilted plants were flattened, fragile and sunken. None of these symptoms was seen in control plants (Plate 1).

There was a relationship between the toxin concentration and the time required for wilting. There was an inverse relationship between toxin concentration and time required for wilting upto 0.125 per cent level. At the lower level of concentration the time required for wilting was more. However, at 0.06 per cent and higher dilution no such inverse relationship between toxin quantity and time to cause symptoms was observed.

Two per cent Sg toxin caused wilting in tomato cuttings within 30 min (Table 1 and Fig.3).

Table 1. Comparative toxicity of *Sclerospora graminicola* crude toxin and its fractions I and II.

Toxin	Toxin concentration (%)							
	2.00	1.00	0.50	0.25	0.12	0.06	0.03	0.05
Crude	30*	60	90	100	120	210	-	-
Fraction-I	30	30	90	120	210	-	-	-
Fraction-II	20	30	60	90	120	150	210	-

* Time in min. required for wilting of tomato cuttings.

- No wilting.

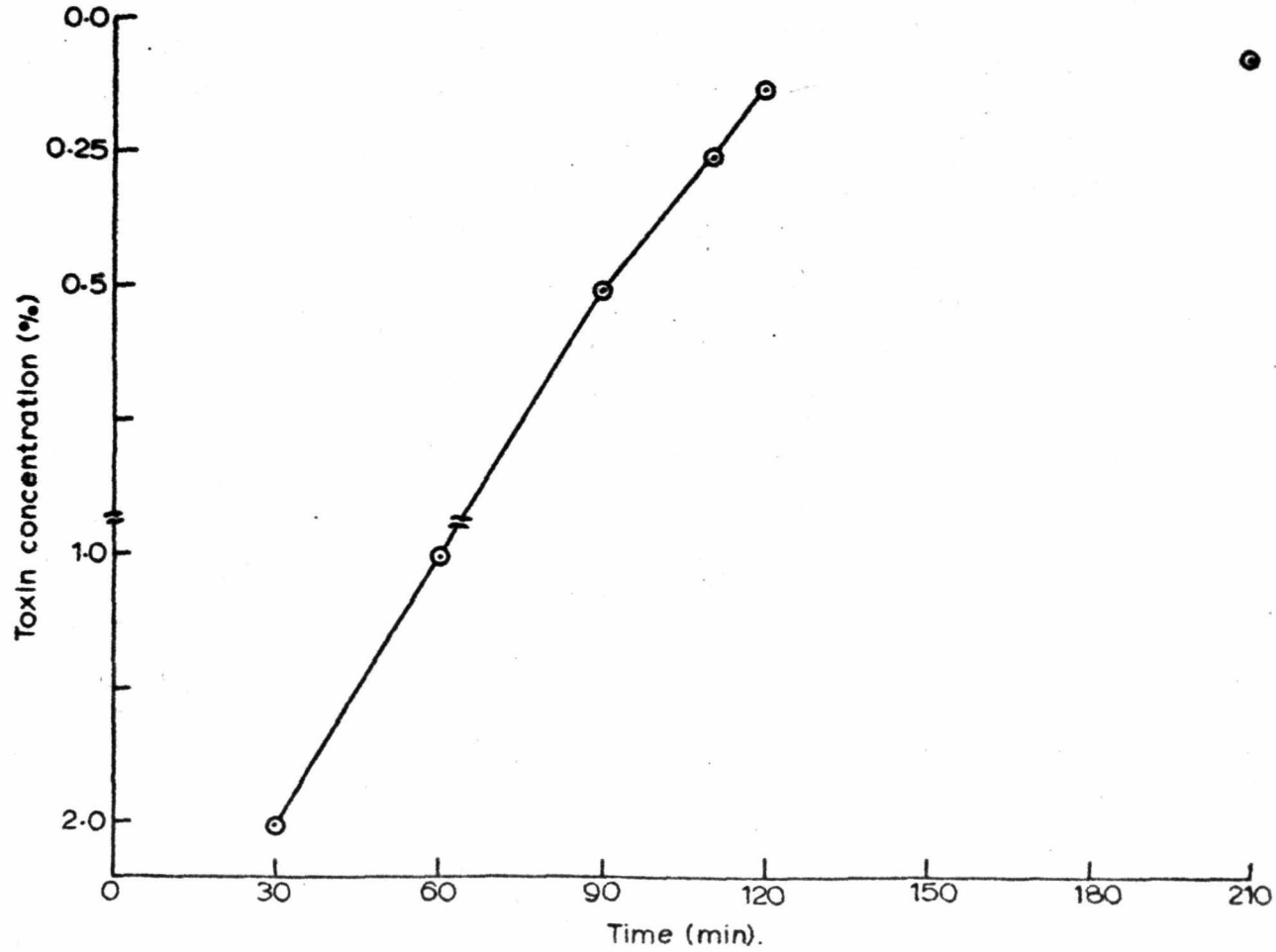


Fig. 3 Relationship between toxin concentration and time required to cause wilting in test plant.

Comparative toxicity of Sg-crude toxin and its purified fractions

Toxicity of Sg-crude toxin and its purified fractions obtained by column chromatography was compared. It showed that the fraction-I was identical in toxicity to that of Sg-crude toxin upto 0.5 per cent level, but at higher dilutions it proved to be less toxic (Table 1). At 0.25 per cent and 0.12 per cent levels the Sg-crude toxin showed symptoms in 100 and 120 min respectively, whereas Fraction-I required 120 and 210 min to cause wilting in the same dilutions. But the fraction-II was more toxic than the Sg-crude toxin and fraction-I. Fraction-II required only 20 min to cause symptoms at 2.00 per cent level and was toxic upto 0.03 per cent level, whereas Sg-crude toxin required 30 min to cause symptoms in tomato plants at 2.0 per cent level and was not toxic at dilutions beyond 0.06 per cent.

Effect of toxin on seed germination, radicle and plumule lengths

Effect of three concentrations viz., 0.5, 1.0 and 2.0 per cent of Sg-toxin on germination of eight different crop seeds and their radicle and plumule lengths was studied. The Sg-toxin affected germination of seeds (Table 2). The direct relationship between toxin concentration and inhibition of seed germination was obtained in case of bajra and ragi seeds. At 2.0 per cent level of the toxin bajra and ragi seeds germination was inhibited by 27 and 12 per cent respectively. At 1.0 per cent level the inhibition of germination in bajra and ragi was 13 and 6 per cent respectively. Similarly, 0.5 per cent toxin inhibited 3 per cent

Table 2. Effect of Sclerospora graminicola toxin on germination, radicle and plumule length of different crop seeds.

Crop	Plant part observed	Control (no toxin)	Toxin concentration (in per cent)		
			0.5	1.00	2.00
Bajra (Pennisetum typhoides)	A*	100.00	97.00	87.00	73.00
	B	4.15	4.66	5.77	6.86
	C	2.57	2.40	2.30	2.40
Ragi (Eleusine coracana)	A	100.00	98.00	94.00	88.00
	B	4.47	3.85	3.83	2.83
	C	2.35	2.28	1.97	1.97
Jowar (Sorghum bicolor)	A	73.00	66.00	66.00	57.00
	B	2.61	2.87	2.90	3.10
	C	2.47	2.34	2.02	1.69
Wheat (Triticum vulgare)	A	86.00	88.00	88.00	84.00
	B	5.80	6.33	6.44	6.45
	C	3.35	3.30	3.20	2.55
Tomato (Lycopersicon esculentum)	A	86.00	84.00	80.00	66.00
	B	6.70	6.15	5.99	5.35
	C	3.50	3.42	3.40	3.33
Green gram (Phaseolus aureus)	A	100.00	76.00	72.00	60.00
	B	2.73	2.76	2.95	3.56
	C	3.17	2.03	1.67	1.33
Black gram (Phaseolus mungo)	A	90.00	88.00	84.00	80.00
	B	2.89	2.80	2.60	1.82
	C	0.90	0.95	1.00	1.00
Navane (Setaria italica)	A	94.00	80.00	76.00	74.00
	B	3.11	2.79	2.83	3.06
	C	2.20	1.52	1.68	2.06

* A = Germination percentage

B = Radicle length (cm)

C = Plumule length (cm)

Plate 2

Effect of Sg-toxin on radicle length of bajra seeds.

1) radicle length of seeds soaked in distilled water (control).

2) in 0.5 per cent Sg-toxin solution.

3) in 1.0 per cent Sg-toxin solution.

4) in 2.0 per cent Sg-toxin solution.

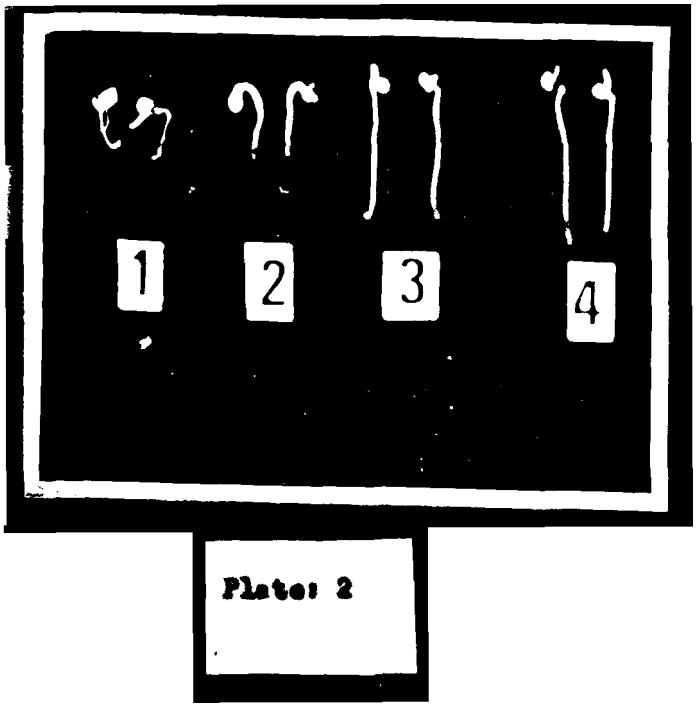


Plate: 2

and 2 per cent seed germination. This clearly indicated that there was an inverse linear relationship between the toxin concentration and seed germination inhibition of crops in particular of bajra and ragi at 2.0 and 1.0 per cent toxin concentrations. In other cases, the germination was affected due to Sg-toxin treatment to a considerable extent but the relationship was not linear (Table 2).

The radicle lengths of various germinating seeds showed a varied response. In majority of the cases the Sg-toxin treatment of seeds before germination, caused increased radicle lengths. Amongst the eight crop seeds studied increased radicle length was noted in five crop seeds viz., bajra, jowar, wheat, green gram and navane. However, the results in bajra seeds were very conspicuous. A direct relationship was observed between the toxin concentrations and the increased radicle lengths (Plate 2). At 0.5 per cent Sg-toxin the increase of radicle length was 0.51 cm whereas, at 1.0 per cent and 2.0 per cent levels, it was 1.52 cm and 2.6 cm respectively. The toxin effect was not directly proportional to the increased growth of radicle but the increase was noticeable. The radicle growth was inhibited in case of ragi, tomato and black gram to some extent as shown in Table 2.

The data in Table 2 also indicated that the plumule length decreased as the toxin concentration increased in all the crop plants tested except

in black gram and navane. There was no direct relationship between the increased toxin concentration and the reduced plumule length. The plumule length of black gram was slightly increased. About 0·1 cm length of plumule was increased at 1.0 per cent and 2.0 per cent toxin concentrations. These results indicated that Sg-toxin adversely affected the plumule i.e., shoot portion of the plant and not the radicle i.e., root portion.

Host specificity test

The host specificity of Sg-toxin was studied by treating the cuttings of various young plants belonging to different families in serially diluted toxin. The results presented in Table 3 revealed that Sg-toxin was not a host-specific toxin and it wilted cuttings of plants belonging to different families. It was observed that in all the cases wherever toxin caused symptoms the highest dilution of the toxin was almost the same, but the time required to cause notable symptoms varied considerably. Amongst the various plants tested paddy was found to be the most resistant followed by green gram and maize in that order of decreasing resistance. The green gram cuttings kept in 2.0 per cent Sg-toxin solution showed curling of leaves after 5 hr and at higher dilutions there was no effect at all. Maize plants showed partial wilting at 0.25 per cent toxin solution which indicated its resistance to Sg-toxin action. The most susceptible plant was found to be tomato. At 0.06 per cent level of Sg-toxin the leaves lost turgidity, drooped down and finally wilted within 210 min time. Bajra took 300 min time at 0.06 per cent level to show the

Table 3. Host-specificity of *Sclerospora graminicola* toxin

Crop	Toxicity	Dilution end point	Time (min)	Remarks
Bajra (*Pennisetum typhoides*)	+	0.06	300	At the beginning leaves lost turgidity, curling and drooping of leaves took place and at higher concentrations of toxin leaves wilted completely and dried.
Tomato (*Lycopersicon esculentum*)	+	0.06	210	Leaves lost turgidity, cuttings drooped down and finally wilted.
Black gram (*Phaseolus mungo*)	+	0.06	360	Leaves lost turgidity and cuttings drooped down.
Green gram (*Phaseolus aureous*)	±	-	-	After five hours only in case of 2.0 per cent toxin concentration curling of leaves was observed.
Wheat (*Triticum vulgare*)	+	0.06	1080	Leaves lost turgidity.
Ragi (*Eleucine coracana*)	+	0.06	360	Wilting of cuttings took place.
Maize (*Zea mays*)	±	0.25	480	Partial wilting was observed, but almost no reaction to toxin.
Paddy (*Oryza sativa*)	-	-	-	No reaction

symptoms. The time required for showing the symptoms and types of symptoms observed in different plants are presented in Table 3.

Effect on bajra plants grown from seeds soaked in Sg-toxin

The seeds of bajra were soaked for about 12 hr in different dilutions of Sg-toxin and then sowed in small pots and plants were grown under green house condition. It was noted that the germination was reduced at 2.0 per cent, 1.0 per cent and 0.5 per cent toxin solutions. At 2.0 per cent level germination was reduced by about 40 per cent whereas, in case of 0.5 and 1.0 per cent levels it was reduced in the range of 5 to 12 per cent. The plants in all the treatments were growing equally well during the early growth stage except showing pale green leaves in toxin treated plants. The differences amongst the treatments were noticeable in two weeks. The leaves showed chlorotic symptoms and became yellow. The curling of the leaves from tip towards base was observed first in the case of plant grown from seeds soaked in 1.0 per cent toxin. One or two days later leaves started becoming fragile and finally wilted. The chlorotic leaves drooped down and started drying from terminal end towards base as seen in Plate 3. The symptoms like chlorosis, curling, drooping, drying and wilting shown by toxin treated plants are some of the major symptoms of the downy mildew disease of bajra. At 0.5 per cent toxin solution similar symptoms but with a lesser intensity were observed. Plants grown from seeds treated in 2.0 per cent toxin showed all these symptoms and in addition showed very low germination. These results indicated that if the Sg-toxin

Plate 3

Bajra plants grown from seeds soaked in Sg-toxin.

1. Control
2. Plants grown from seeds soaked in 0.5 per cent Sg-toxin solution
3. 1 per cent Sg-toxin solution.
4. 2 per cent Sg-toxin solution.

Plate: 3

was allowed to be absorbed in sufficient quantity by the germinating bajra seeds most of the downy mildew symptoms can be mimicked. It suggested that Sg-toxin must have some role in the causation of symptoms like chlorosis, curling and drying of leaves.

Effect on the growth of microorganisms

The effect of Sg-toxin on the growth of various microorganisms was tested by using 1.0 per cent, 2.0 per cent and 4.0 per cent Sg-toxin solution. Sg-toxin did not affect the growth of any of the 23 micro-organisms, belonging to different groups viz., algae, actinomycetes, bacteria and fungi (Table 4).

Histochemical changes

The investigation regarding morphological and histochemical changes in Sg-toxin treated and control germinating seeds revealed that there were no significant changes in radicle, coleoptile or embryo, when observed under light microscope.

Leaves

In bajra the leaf is dorsiventrally differentiated. The lower epidermis is having more number of stomata than the upper one. The mesophyll cells contain discoid plastids. Both large and small veins have sheaths made up of larger parenchymatous cells. The midrib region has 8-10 veins.

Table 4. Effect of *Sclerospora graminicola* toxin on the growth of microorganisms.

Organism	Growth inhibition at different toxin concentration (per cent)		
	1.0	2.0	4.0
Aerobacter aerogens	-	-	-
Azotobacter chroococcum	-	-	-
Bacillus subtilis	-	-	-
Escherichia coli	-	-	-
Klebsiella spp.	-	-	-
Nocardia spp.	-	-	-
Pseudomonas solanacearum	-	-	-
Rhizobium spp.	-	-	-
Sarcina lutea	-	-	-
Streptococcus aureus	-	-	-
Actinomyces spp.	-	-	-
Streptomyces spp.		-	-
Saccharomyces cerevicese	-	-	-
Chlorella spp.	-	-	-
Alternaria spp.	-	-	-
Aspergillus spp.	-	-	-
Chaetomium spp.	-	-	-
Fusarium spp.	-	-	-
Ganoderma spp.	-	-	-
Helminthosporium oryzae	-	-	-
Helminthosporium sacchari	-	-	-
Phytophthora arecae	-	-	-
Pyricularia spp.	-	-	-

Plate 4

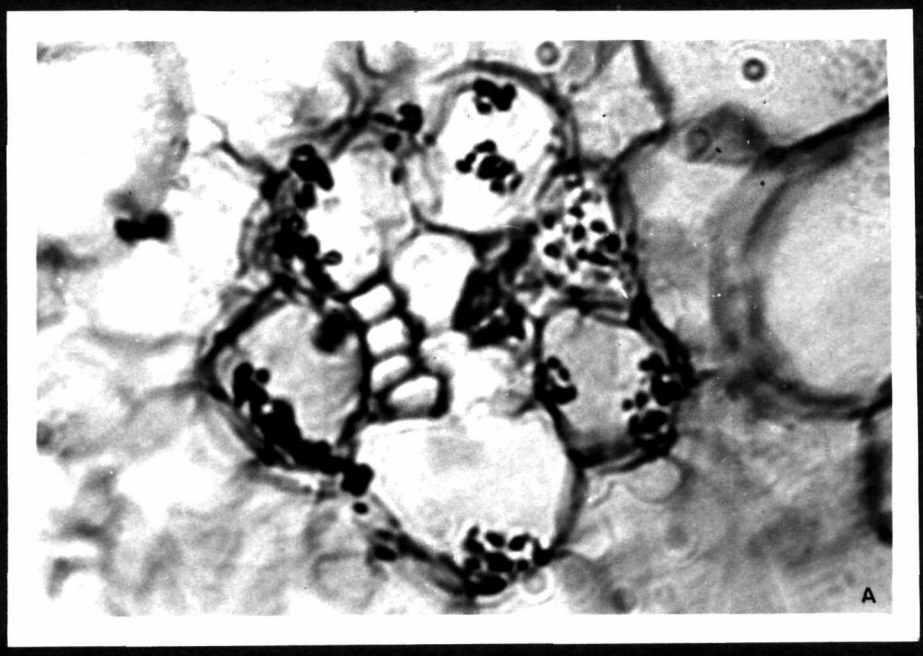
Effect of Sg-toxin on insoluble polysaccharides (starch) content of bajra leaf cells.

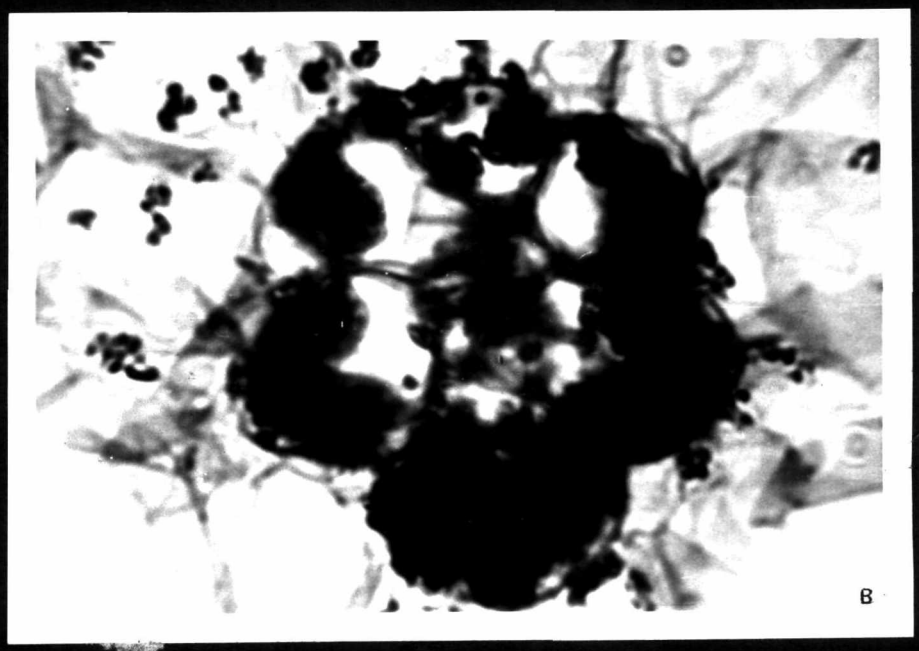
A = Starch content of normal bajra leaf cells (Plants grown from control seeds).

B = Starch content in I stage of symptoms*

C = Starch content in III stage of symptoms*

* Seeds were treated in 2.0 per cent Sg-toxin solution before raising the plants.





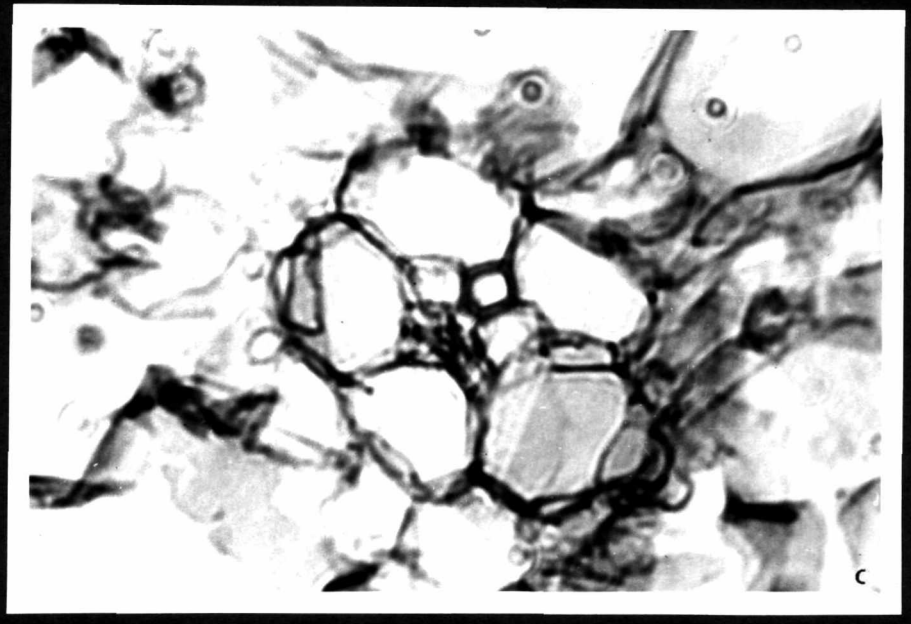


Plate: 4

Insoluble polysaccharides (Starch)

The presence of insoluble polysaccharides in leaf tissues were detected by following the periodic Acid-Schiffs (PAS) test. In control leaf sections only the bundle sheath cells contained small PAS positive granules. No PAS positive granules were observed in any of the other mesophyll or epidermal cells (Plate: 4).

In the sections of stage 1 and 2 plant leaves from Sg-toxin treated seeds there were no significant changes in insoluble polysaccharide contents. However, the increased number of polysaccharide granules in bundle sheath cells from stage 1 leaves was noted and at stage 2 the lessening of the PAS positive granules in the bundle sheath cells was observed. At stage 3 significant changes were observed. All the accumulated starch grains were found to have disappeared (Plate: 4).

MGP test for DNA and RNA

The studies related to DNA and RNA in cells were conducted by following the Methyl Green Pyronin (MGP) test as mentioned earlier. In the control it was noted that the epidermal cells, mesophyll cells and also the bundle sheath cells did not contain much RNA. The plastids were very faintly positive for pyronine-G indicated that very little amount of RNA was present surrounding them. The nuclei which were smaller than the plastids were found to be brightly stained for DNA and nucleoli were deeply stained for RNA.

Plate 5

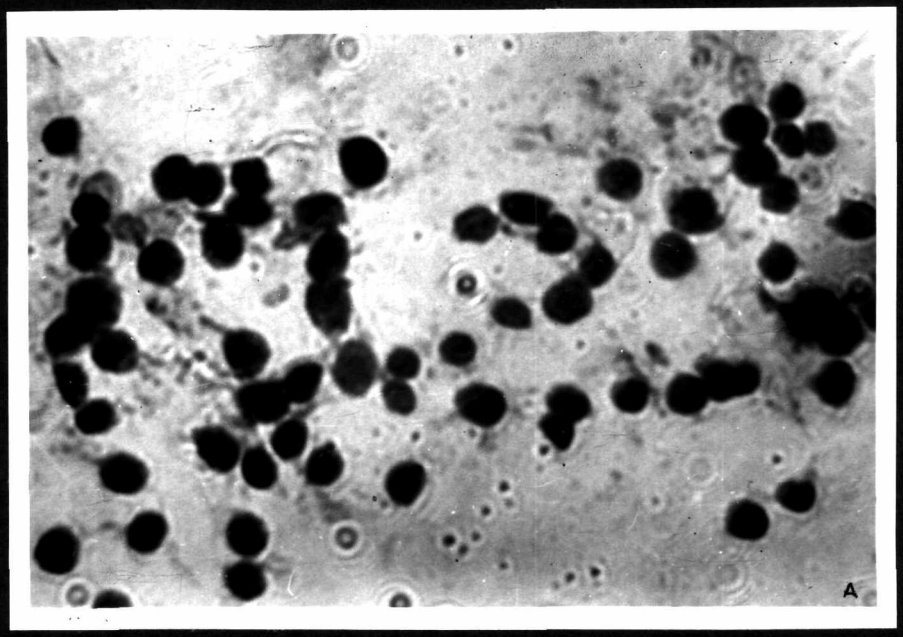
Effect of Sg-toxin on protein content of bajra leaf cells.

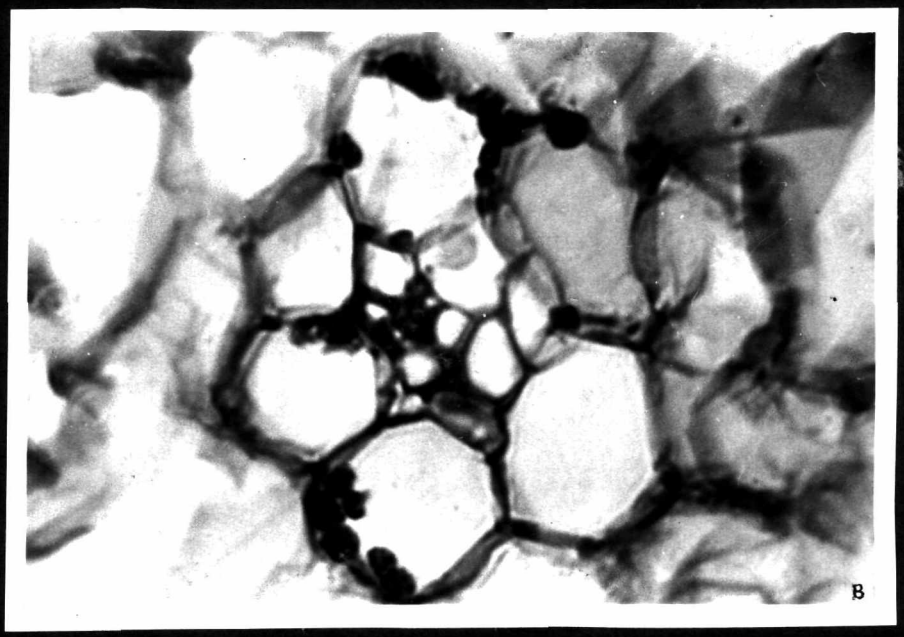
A = Large number of protein grains scattered throughout the leaf cells of normal bajra plant (Plants grown from control seeds).

B = Reduced number of protein grains observed during I stage of symptoms*.

C = Absence of protein grains observed during III stage of symptoms*.

* Seeds were treated in 2 per cent Sg-toxin solution before raising the plants.





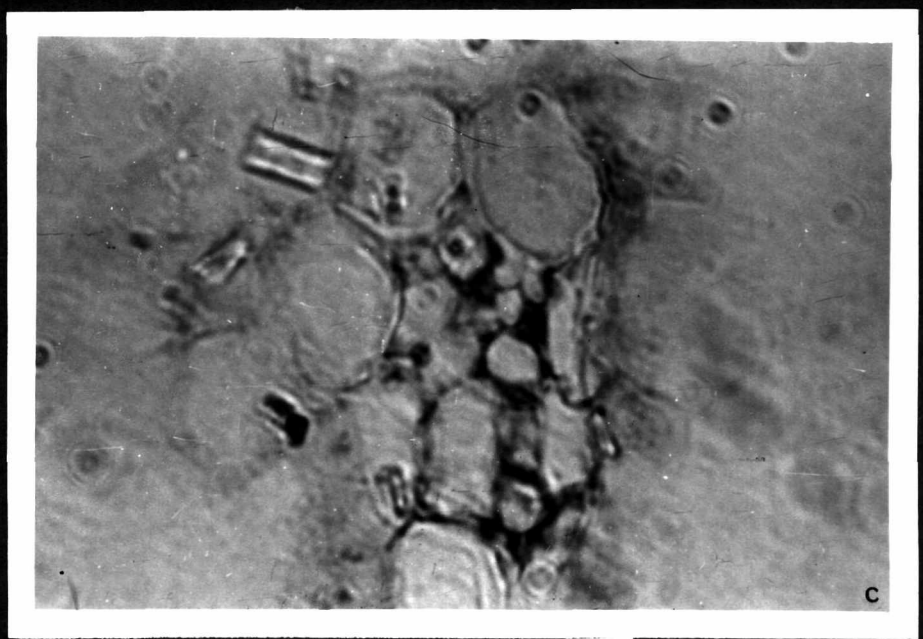


Plate: 5

The results of the samples taken from stage 1 and 2 indicated that there were no significant changes in RNA content of the nucleus and cytoplasm. The nuclei were found to be stained for DNA. At stage 2 the cytoplasm around the plastids stained very faintly for RNA. At stage 3 in most of the cells the nuclei were found to be disappeared. The presence of plastids could not be detected. Most part of the DNA and RNA had completely degraded.

Proteins

The data on proteins in leaves as determined by mercuric bromophenol blue method revealed that in control tissues the protein positive bodies were discoid in shape and they were larger in bundle sheath cells. Their distribution was found to be more in the area nearing the bundle sheaths than in the mesophyll cells (Plate: 5).

At stage 1 the protein positive bodies were less brightly stained as compared to control. The shape was getting distorted. Both these facts indicated the degradation of plastid material (Plate: 5). It was observed from the sections of leaves at stage 2 that protein positive bodies became still smaller in size and their stainability was faint. They had distorted outlines. Some of the bundle sheath cells had almost completely lost those bodies. At stage 3 it was noted that all the protein positive bodies found in mesophyll cells and bundle sheath cells have been completely disappeared (Plate: 5).

Lipids

There were no lipids either in control or treated plants.

Serology

The antiserum against the Sg-toxin was prepared as mentioned in Materials and Methods. The titer of the antiserum was determined by following the microprecipitin test. The highest dilution of the antiserum which showed the precipitation was taken as the titer of the antiserum and it was found to be 1:1280. These results revealed the antigenic nature of the Sg-toxin.

From the results of immunodiffusion test (Fig.4) it was observed that Sg-crude toxin and its purified fractions I and II were serologically identical. The precipitation bands did not cross each other in any case. Two precipitation bands appeared, a thick one near the central well which contained antiserum and a thin single band near the peripheral well containing the antigen. These results suggested the presence of two types of molecules in samples viz., one having a smaller size which could diffuse fast towards a central well and the other a bigger size molecule which moved slower towards the central well.

The temperature studies for incubation of immunodiffusion plates indicated that 4°C and room temperature were suitable temperatures but not 37°C. The plates incubated at 4°C showed clear precipitation bands

Fig. 4. Serological relationship between the Sg-crude toxin and its fractions I and II.

S = Normal physiological saline (0.85%)

C = Sg-crude toxin

I = Fraction I

II = Fraction II

NS = Normal serum

AS = Pooled antiserum

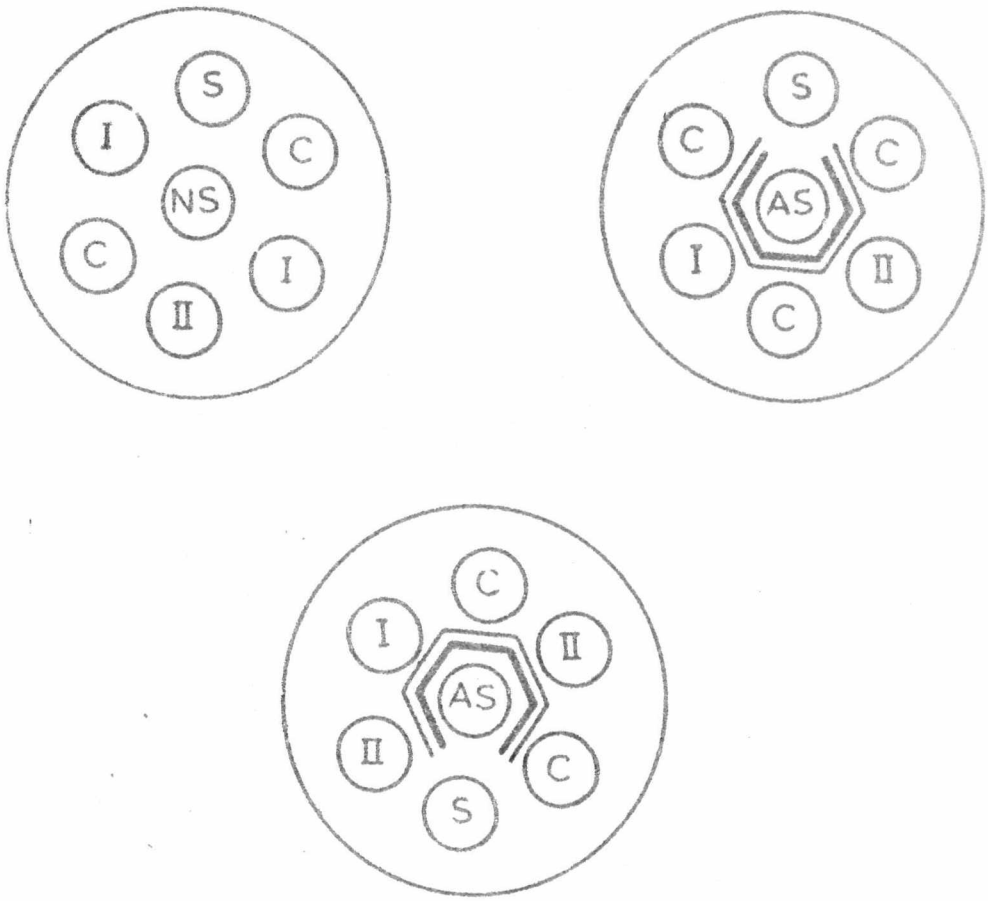


FIG. 4 SEROLOGICAL RELATIONSHIP BETWEEN THE Sg-CRUDE TOXIN AND IT'S FACTIONS I AND II.

after 24 hr whereas, for those incubated at room temperature 48 hr time was required. No bands were seen in the plates incubated at 37°C even after 48 hr. It was also noted that 0.01 M PO_4 buffer (pH = 7.00), normal saline (0.85 per cent) or distilled water could be used for preparing agar for immunodiffusion techniques. The antigen could be dissolved in either of the solvents i.e., PO_4-buffer or normal saline. Both solvents gave equally good results. No precipitation bands could be seen in those plates where normal serum was placed instead of antiserum and saline instead of antigen (Fig: 4).

Inactivation of Sg-toxin

Effect of storage

The results from this experiment indicated that completely dried Sg-toxin could be stored in either desiccator or refrigerator for a long time, as long as 18 months without loosing its activity. It indicated that Sg-toxin was a stable compound and could be easily stored for a sufficiently long period. The samples drawn from both the sets at various intervals and the last sample after 18 months were found to be equally toxic as that of freshly isolated toxin indicated that the activity of Sg-crude toxin was not affected even during the storage period of 18 months.

Effect of temperature

The test on the effect of temperature on the activity of Sg-toxin indicated that Sg-toxin was quite stable upto 95°C but it lost its activity at 97°C and above if heated for 10 min. The samples heated for 10 min upto 95°C showed the same toxicity as that of control (not exposed to any of the test temperatures), but the sample which was heated at 97°C for 10 min in water bath did not show any toxicity indicating that it had lost the activity.

Effect of partial acid hydrolysis

In this experiment Sg-toxin was exposed to 1 N H_2SO_4 for various lengths of time ranging from 15 to 90 min and the toxicity of each sample drawn at various intervals was tested as mentioned under Materials and Methods. It was noted that at 15 min interval the activity of Sg-toxin was reduced to some extent i.e., it caused wilting of plant cuttings in 180 min whereas the untreated control caused wilting in 150 min. After 20 min exposure it took 200 min for the treated toxin to cause symptoms in test plant cuttings (Fig.5). The toxin samples exposed for more than 20 min did not cause any visible symptoms in test plants, indicating that the Sg-toxin had lost its activity. This experiment clearly indicated that exposure of Sg-toxin to 1 N H_2SO_4 for shorter time intervals reduced its activity and if exposed for more than 20 min it lost its activity completely.

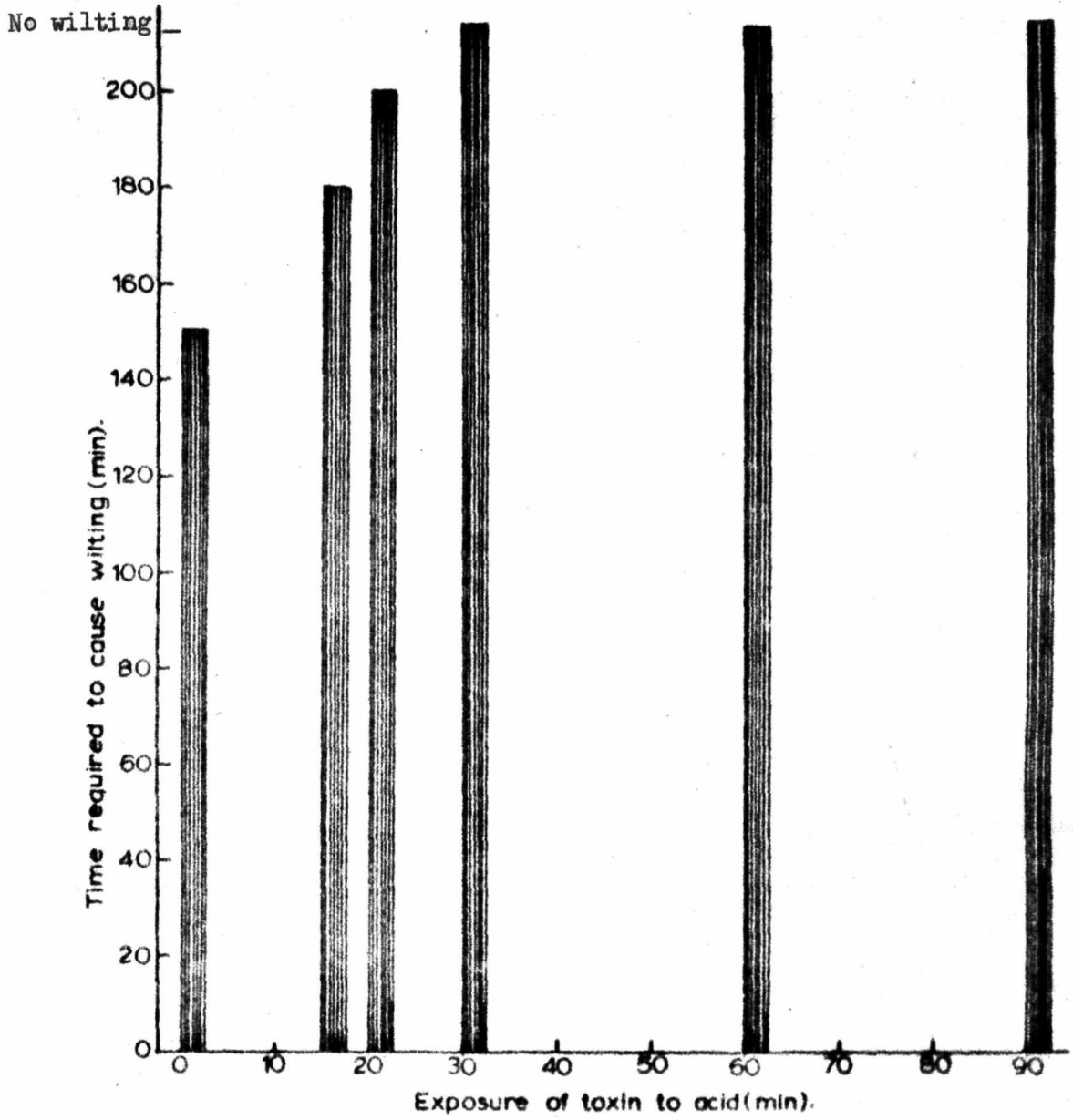


Fig. 5 Relationship between partial acid hydrolysis in 1 N H_2SO_4 of Sg-toxin and time required to cause wilting.

Physical characteristics

The Sg-crude toxin when completely dried was dark brown and crystalline in nature (Table 5). It was highly hygroscopic and when exposed to room environment it readily absorbed moisture, from atmosphere and formed a clump. In solution Sg-crude toxin was slightly viscous in nature. Fraction-I was faint yellow in colour and fraction-II was dark brown in colour. Both fractions were hygroscopic in nature.

Column chromatography

Small quantity (15 mg) of Sg-crude toxin was dissolved in 2 ml of distilled water and loaded on the Sephadex G-200 gel column. The toxin was eluted with distilled water and fractions of 3 ml were collected separately as mentioned earlier. The absorbance was read by using Spectronic-20 at 540 mμ. The elution volume was plotted against the absorption to get a graph as plotted in Fig.6.

By column chromatography Sg-toxin was fractionated in to two fractions. Two peaks were obtained as depicted in Fig.6. One at an elution volume of 12 ml and the other one at an elution volume of 24 ml. These two fractions were air dried separately and total recovery of the compound and their proportional recovery was calculated. The total recovery of Sg-toxin from column was 78.00 per cent and 22.00 per cent of it was lost during the process of purification. The fraction-I

Table 5. Physical properties of Sclerospora graminicola and its fractions I and II.

Property	Crude toxin	Fraction I	Fraction II
Colour	Dark brown	Faint yellow	Dark brown
Molecular weight	-	2,73,000	61,630
Intrinsic viscosity (decilitre/g)	0.122	0.069	0.047
pH	6.98	6.70	6.90

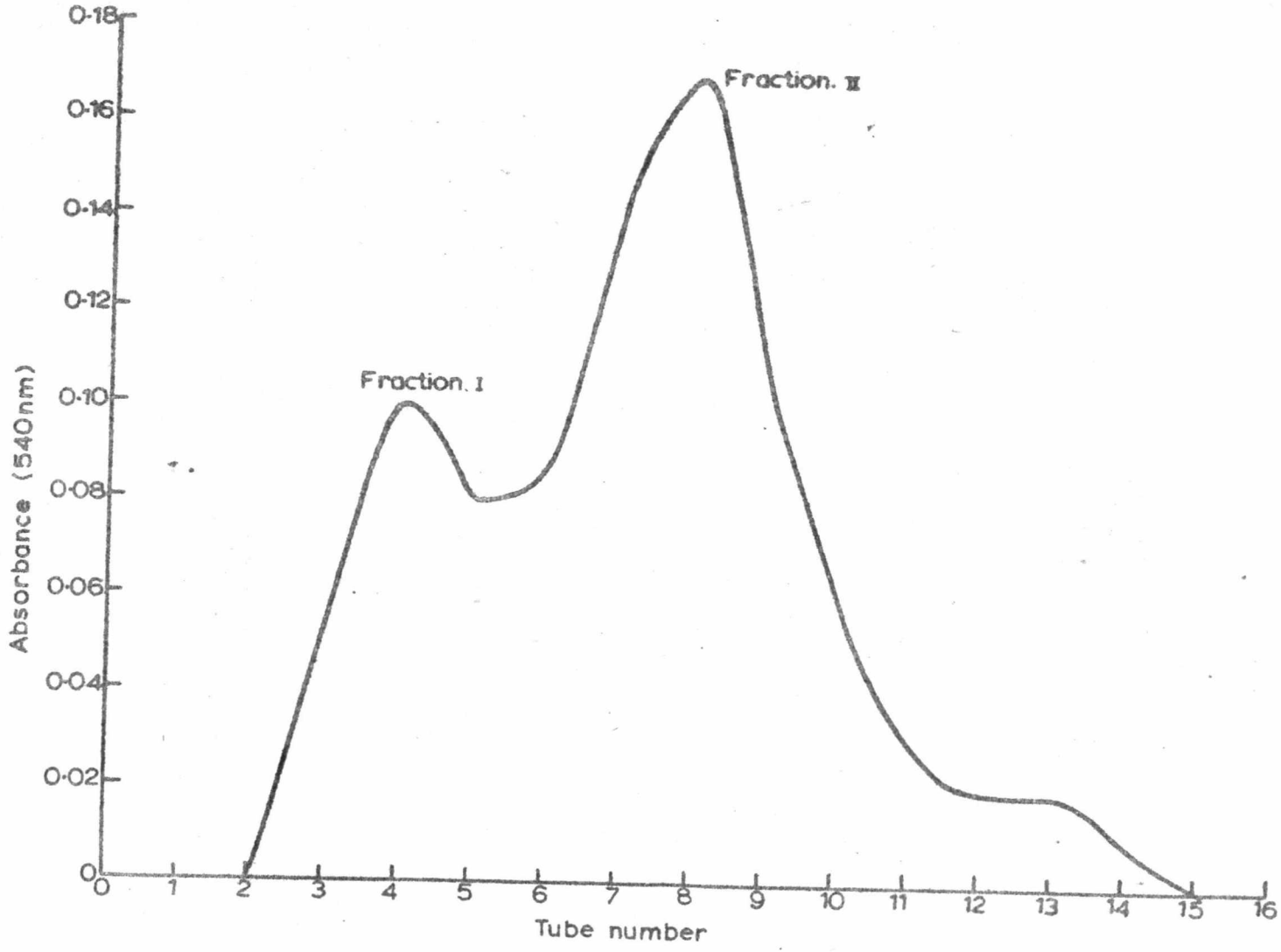


Fig. 6 Fractionation of the Sclerospora graminicola toxin Preparation by Sephadex-G-200 gel Chromatography. Fractions of 3ml were collected. Absorbance at 540 nm.

constituted 27.0 per cent and the fraction-II constituted 51.0 per cent giving total recovery of 78.0 per cent through column chromatography (Fig.2). It could be seen from these results that fraction-II was almost double in weight that of fraction-I.

Molecular weight determination

The molecular weights of Sg-toxin fractions were calculated by following the method described earlier. The elution volume of the fraction-I was 12 ml and that of fraction-ii was 24 ml. The estimated molecular weight of fraction-I was 2,73,200 and that of fraction-II was 61,630. The results are presented in Table 5.

Specific viscosity determination

The specific viscosity of Sg-crude toxin and its purified fractions was determined and from that the intrinsic viscosity of the Sg-crude toxin and its purified fractions as noted in Table 5 was obtained by calculations. The intrinsic viscosity of the Sg-crude toxin was 0.122 decilitre/g and that of fractions-I and II was 0.089 and 0.047 decilitre/g respectively. It could be seen from these results that as the molecular weight of fraction decreased the intrinsic viscosity was also reduced.

Biochemical properties

pH determination

It was noted that the Sg-crude toxin had pH of 6.98 and the

Plate 6

Electrophoretic movement of Sg-toxin on paper.

A = Sprayed with ninhydrin.

B = Developed with Trevelyan reagents.

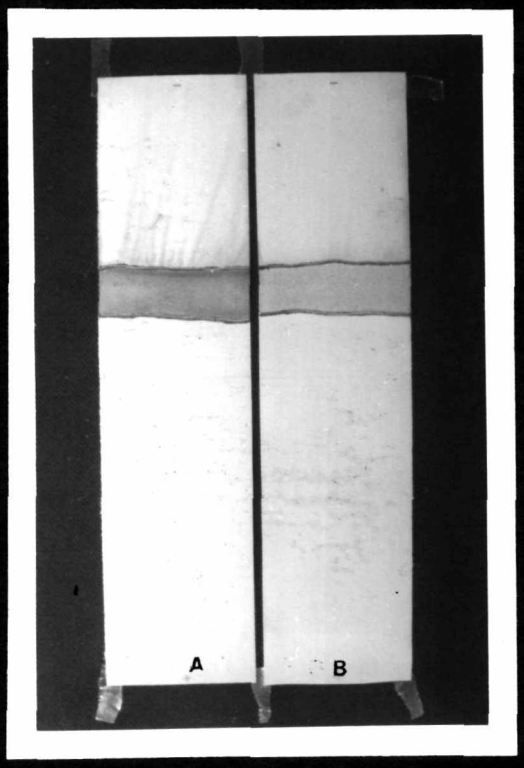


Plate: 6

fractions-I and II had pH of 6.70 and 6.90 respectively. These results indicated that pH of Sg-crude toxin was near neutrality but its purified fractions were slightly acidic in nature. The fraction-I was slightly more acidic having pH of 6.70 as compared to fraction-II which had pH of 6.90.

Electrophoretic studies

Electrophoretic properties of the Sg-crude toxin were studied by following the paper electrophoresis as mentioned earlier. The results indicated that the Sg-toxin consisted of only one fraction on the basis of electrophoretic mobility. The compound moved towards cathode which indicated that it was cationic in nature. The electrophoretically moved spot gave positive test with ninhydrin as well as reagents of Trevelyan method (Plate: 6).

Acid hydrolysis and ion-exchange chromatography

The Sg-crude toxin and its purified fractions were acid hydrolysed at 100-110^{0}C for 12 hr and then neutralized. These samples were fractionated into an anionic, cationic and neutral fractions. Each fraction was separately dried and weighed to calculate total recovery and proportion of each fraction to other. It was found that the total recovery of Sg-crude toxin was 52 per cent and 48 per cent was lost during hydrolysis and neutralization. The anionic, cationic and neutral fractions of Sg-crude toxin were in the proportion of 5 : 3 : 5 on weight

basis. The results revealed that total recovery of fraction-I and II after hydrolysis was 73 and 64 per cent respectively. The fraction-I and II contained three fractions viz., anionic, cationic and neutral in the proportions of 3 : 5 : 3 and 2 : 9 : 5 respectively. It was found that neutral fraction formed the largest proportion among three fractions in case of Sg-crude toxin and its purified fractions. The neutral fraction was followed in proportions by cationic and anionic fractions in Sg-crude toxin and its purified fractions on weight basis.

Sugar analysis

The neutral fraction of Sg-crude toxin and its fractions I and II were analysed for their sugar contents by following descending paper chromatography. The results regarding qualitative analysis for sugar contents of Sg-crude toxin and its purified fractions are presented in Table 6. The results indicated the presence of four sugars in Sg-crude toxin and three each in fraction-I and II. The Sg-crude toxin contained arabinose, glucose and two unknown with the Rg values of 0.42 and 0.58. The fractions I and II contained arabinose and glucose and one unknown sugar in each fraction having Rg value of 0.53 and 0.60 respectively.

The quantification of arabinose and glucose from Sg-crude toxin and its purified fractions was done by following Nelson's method. The results indicated that Sg-crude toxin contained arabinose 0.036 per cent and

Table 6. Sugar and organic acid composition of *Sclerospora graminicola* toxin and its fractions I and II.

Toxin	Sugar	Rg value*	Organic acid	Rg value*
Crude	Arabinose		D-galacturonic acid	
	Glucose		Unknown	1.15
	Unknown	0.58	Unknown	0.75
	Unknown	0.42	Unknown	0.60
Fraction-I	Arabinose		Unknown	1.12
	Glucose		Unknown	0.78
	Unknown	0.53	Unknown	0.52
Fraction-II	Arabinose		Unknown	1.11
	Glucose		Unknown	0.80
	Unknown	0.60		

* Rg values with reference to Glucose

*Rg values with reference to D-galacturonic acid

glucose 0.016 per cent by weight, whereas fraction-I and II contained arabinose and glucose 0.015 per cent, traces and 0.004 per cent traces respectively.

Sugar acid analysis

The results of qualitative analysis of sugar acids of Sg-crude toxin and its purified fractions are presented in Table 6. These results indicated that Sg-crude toxin contained four sugar acids out of which one was identified as D-galacturonic acid and the remaining three were unknown with the Rg values of 0.60, 0.75 and 1.15. The fraction-I contained three sugar acids which were unidentified with the Rg values of 0.52, 0.78 and 1.12. The fraction-II contained only two sugar acids and both were unknown with the Rg values of 0.80 and 1.11.

Amino acid analysis

The amino acid composition of Sg-crude toxin and its purified fractions was determined by analysing the samples in automatic amino acid analyser (Figs.7, 8 and 9). The quantitative estimation of each known amino acid was also done and the results are presented in Table 7. It was noted that Sg-crude toxin contained about 7.99 per cent total amino acids on weight basis. In all it contained 24 amino acids amongst which five were basic amino acids and the remaining 19 were neutral and acidic amino acids. Amongst five basic amino acids two were unknown and of the remaining 19, 5 were unknown. The aspartic acid content of 2.6834

Table:7 Amino acid composition of Sclerospora graminicola crude toxin and its purified fractions.

Sl. No.	Amino acid	Crude toxin	Fraction I	Fraction II
		(Mg. per 100 mg. of toxin)		
	Basic amino acids			
1	Lysine	0·4912	0·1095	0·1753
2	Histidine	0·2792	0·0465	0·0933
3	Arginine	0·3764	0·0785	0·1393
4	Unknown	+	+	+
5	Unknown	+	+	−
	Neutral and acidic amino acids.			
6	Aspartic acid	2·6834	0·2195	0·9904
7	Threonine	0·3288	0·1250	0·1716
8	Serine	0·3658	0·1575	0·1764
9	Glutamic acid	1·2182	0·2430	0·5828
10	Proline	0·4006	0·2075	0·1244
11	Glycine	0·3874	0·1350	0·2612
12	Alanine	0·4384	0·1470	0·2460
13	Cystine	0·1298	Traces	0·0432
14	Valine	0·2390	0·1055	0·1124
15	Methionine	0·0358	Traces	0·0360
16	Isoleucine	0·1418	0·0395	0·0632
17	Leucine	0·2362	0·0790	0·1576
18	Tyrosine	0·0870	Traces	0·0436
19	Phenylalanine	0·1586	Traces	0·0792
20	Unknown	+	+	+
21	Unknown.	+	+	+
22	Unknown	+	−	+
23	Unknown	+	−	+
24	Unknown	+	−	−

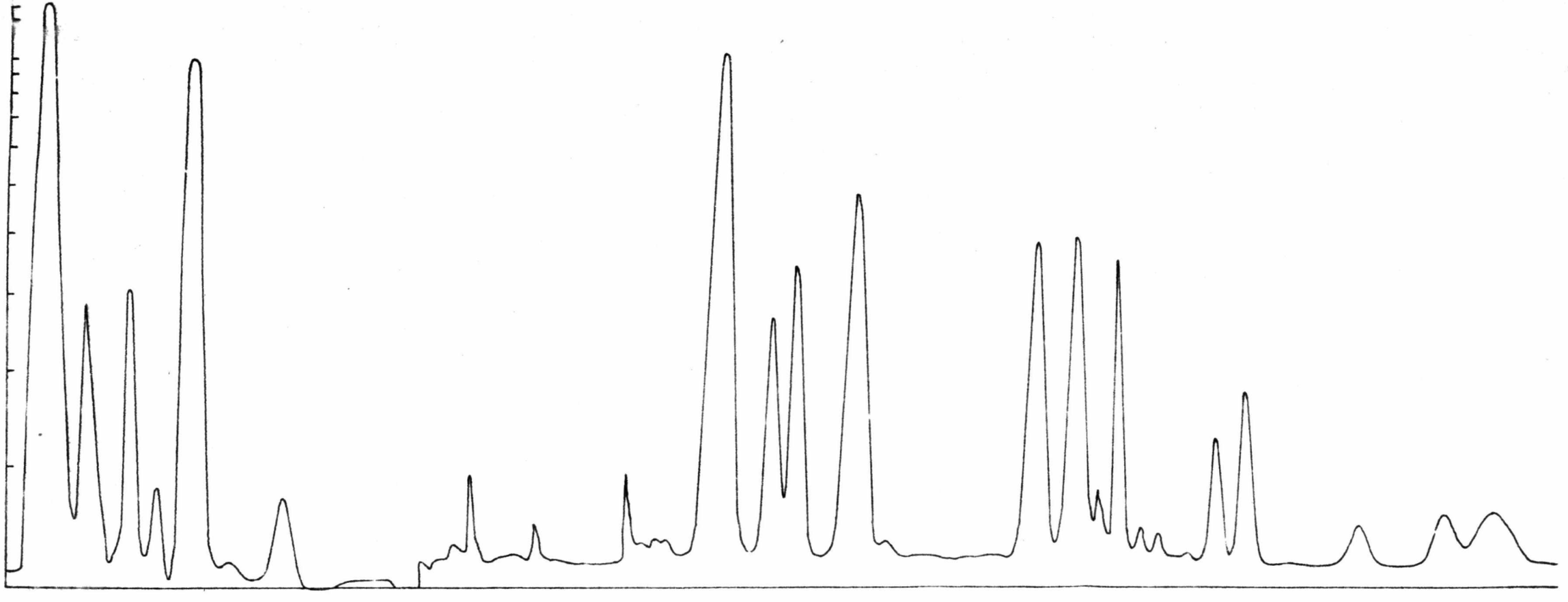


FIG. 7 QUALITATIVE AND QUANTITATIVE ANALYSES OF AMINO ACIDS CONTAINED IN THE CRUDE TOXIN.

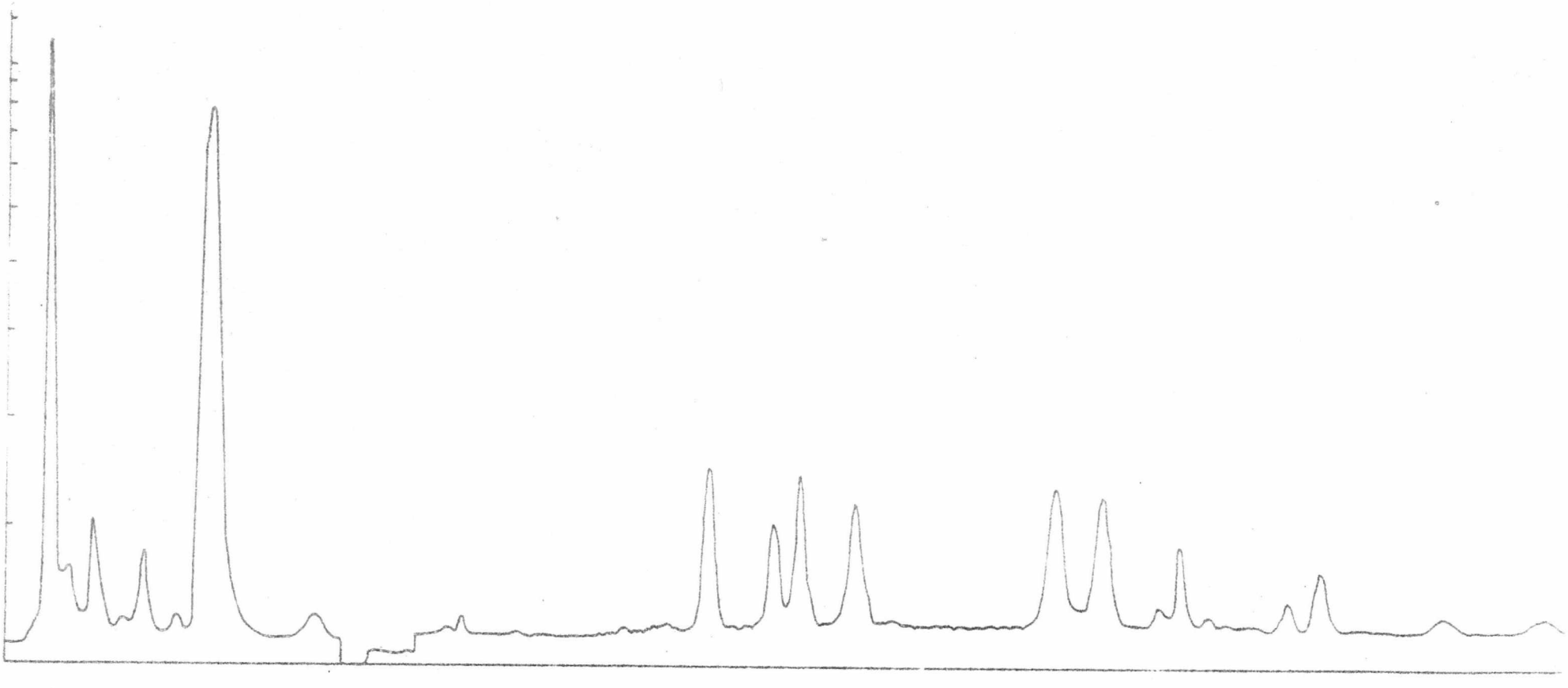


FIG. 8 QUALITATIVE AND QUANTITATIVE ANALYSES OF AMINO ACIDS CONTAINED IN THE FRACTION I

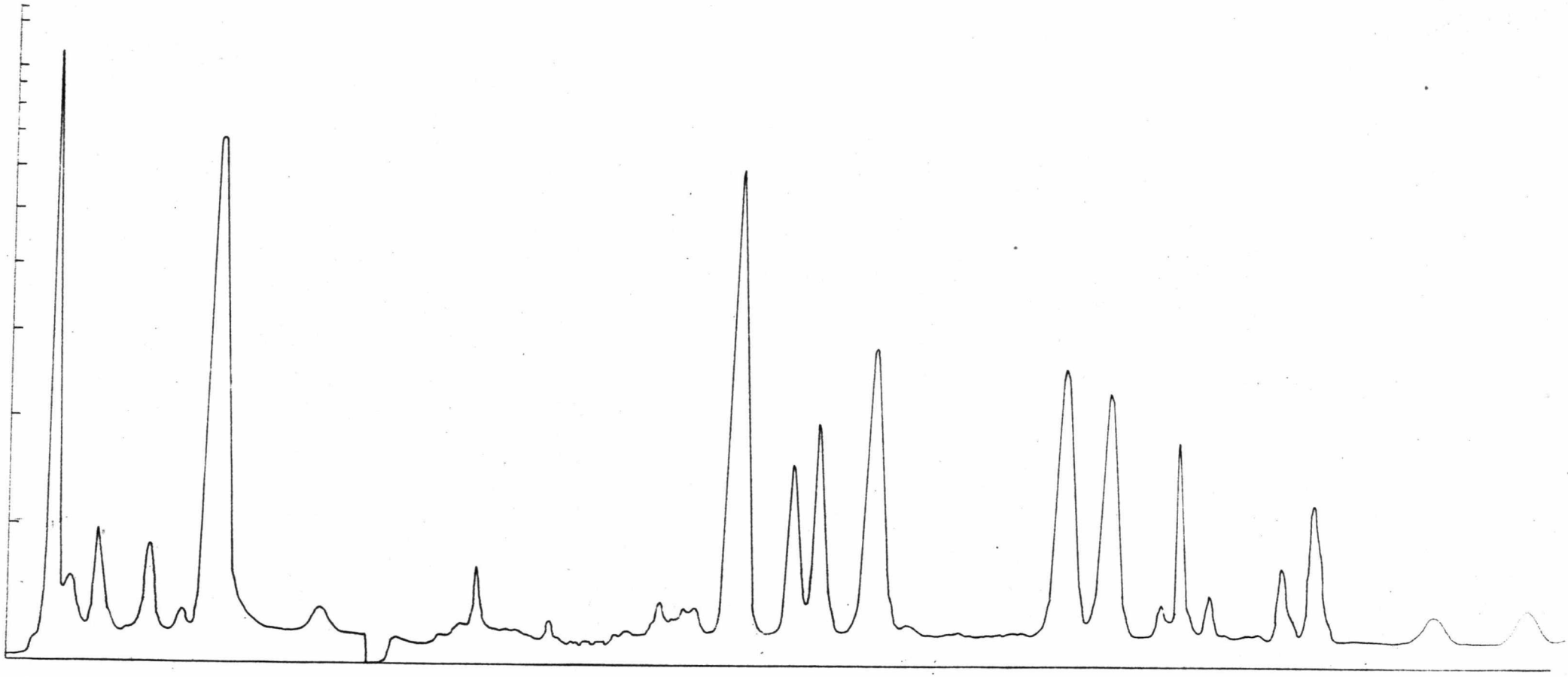


FIG. 9 QUALITATIVE AND QUANTITATIVE ANALYSES OF AMINO ACIDS CONTAINED IN THE FRACTION. II

per cent in Sg-crude toxin was the highest value amongst all the amino acids and followed by lysine, alanine, proline, glycine, arginine and several others as indicated in Table 7.

The calculations revealed that the recovery of amino acids in fraction-I and II from Sg-crude toxin was not cent per cent. About 30 per cent amino acids were lost during the process of purification and hydrolysis. The fraction-I contained 1.68 per cent and fraction-II contained 3.49 per cent total amino acids on weight basis. In general, fraction-II contained all the amino acids in higher quantities than those in fraction-I except for proline which was higher in fraction-I. The fraction-I contained, in all, 21 amino acids whereas, fraction-II contained 22 amino acids. Fraction-I contained five basic amino acids as that of Sg-crude toxin, whereas fraction-II contained only four basic amino acids. The fraction-I contained 16 neutral and acidic amino acids out of which only two were unknown, whereas, fraction-II contained 18 neutral and acidic amino acids out of which four were unknown. Fraction-I contained cystine, methionine, tyrosine and phenylalanine in traces but fraction-II contained considerable quantities of these amino acids. Fraction-I contained glutamic acid in highest quantity i.e., 0.243 per cent whereas fraction-II contained aspartic acid in highest quantity (0.99 per cent). These results clearly indicated that fraction-II was more closely related to Sg-crude toxin in amino acid composition than fraction-I.

Table 8. Elemental analysis of Sclerospora graminicola crude toxin and its purified fractions.

Toxin	Carbon (%)	Hydrogen (%)	Oxygen (%)	Nitrogen (%)	Empirical formula
Crude toxin	27.06	3.99	64.35	4.60	$C_7H_{12}O_{13}N$
Fraction-I	-	-	-	1.03	-
Fraction-II	14.62	2.35	80.78	2.25	$C_8H_{15}O_{32}N$

- analysis was not done

Elemental analysis

The Sg-crude toxin and its purified fractions were analysed for their carbon, hydrogen, nitrogen and oxygen contents. From the results presented in Table 8, it could be seen that Sg-crude toxin contained 4.60 per cent total nitrogen, whereas fraction-I and II contained 1.03 and 2.25 per cent total nitrogen respectively. It was calculated that the recovery of total nitrogen in fraction-I and II from Sg-crude toxin was only 72.85 per cent and the remaining quantity of 27.15 per cent was lost during the process of purification. From the percentage values of C, H, O and N the empirical formulae of Sg-crude toxin and its purified fraction were calculated on the basis of one atom of nitrogen. The empirical formulae of Sg-crude toxin and its fraction II were $C_7H_{12}O_{13}N$ and $C_8H_{15}O_{32}N$.

DISCUSSION

V. DISCUSSION

Inoculation of bajra plants with Sclerospora graminicola indicated that sporangial inoculation was superior over oospore inoculation which resulted in early and more infection. Safeeulla (1976) reported that for obtaining higher percentage of infected plants, it was necessary to allow the oospore material to weather in the soil for at least four weeks. Bajra plants of 6-7 cm height and 1-2 weeks old are optimum for inoculation and show the preliminary symptoms of chlorosis yellowing and downy growth. Younger and smaller plants directly wilt on inoculation. The organism could be easily maintained on bajra plants grown in sick soil. The oospore of S.graminicola survived in soil for several years (Safeeulla, 1976).

The toxic compounds were isolated from the infected bajra plants (Fig.1) but not from the healthy plants. Toxic compounds have been recently reported from obligate parasites. Millerd and Scott (1955) reported a toxin from powdery mildew infected barley plants. Silverman (1960) reported another toxin from infected wheat stem rust fungus. Recently, Rai (1977b) reported the production of a non-specific toxic compound in grape leaves infected by Plasmopara viticola. About 0.50 per cent toxic compound was present in infected bajra plant (Fig.2). Detection of the toxin becomes a problem because of its occurrence in minute amounts.

Toxicity of the compound was tested by using tomato, and bajra plant cuttings (Table 1, Fig.3) as suggested by various workers (Hodgson et al., 1947, 1949 and Nesf-Rath, 1972). Rai (1977b) used tomato cuttings and grape leaves to test the toxicity of the compound produced by Plasmopara viticola. Bioassay studies revealed that the dilution end point of Sg-toxin was 0.06 per cent. The recovery of the toxin from infected plants and its dilution end point suggested that S.graminicola produced a large amount of the toxic compound in infected plants as compared to that necessary to cause disease symptoms. S.graminicola produced sufficient amount of toxic compound in infected plants to cause disease symptoms and such a toxic compound could not be isolated from healthy plants. These results fulfilled the criteria for vivotoxin suggested by Dimond (1955). The studies on toxin concentration and time relationship revealed that there was inverse relationship between these two factors. As the toxin concentration increased the time required to cause wilting was decreased. The symptoms were caused by 2.0 per cent toxin solution in 30 min which indicated that the toxin was quite potent and highly toxic in activity.

The comparative toxicity of Sg-toxin and its fractions I and II (Table 1) revealed that fraction-I was identical in toxicity to Sg-crude toxin upto 0.5 per cent level but at higher dilutions there was no similarity. The fraction-II was more toxic to plants. These results can be explained on the basis of up-take of toxin by plants. The molecular

weight (Table 5) of the fraction-II was lower than that of fraction-I and hence might be it translocated faster in the plants.

Sg-toxin inhibited seed germination as shown in Table 2. The most conspicuous results were noted in case of bajra and ragi seeds wherein direct relationship between the toxin concentration and inhibition of seed germination was noted at 2.0 per cent and 1.0 per cent toxin concentration. In other cases the germination was inhibited to a considerable extent but even at higher levels of toxin the relationship was not linear (Table 2). These results suggested that bajra and ragi seeds were affected more by Sg-toxin as compared to other seeds. This might be due to the partial specificity of Sg-toxin. S.graminicola affects bajra and it can be grown on ragi callus (Safeeulla, 1976). It appears reasonable to expect that seeds absorb Sg-toxin in soil and loose viability. This is of biological interest because S.graminicola is considered as a soil borne pathogen, if germinating oospores produce the toxic principle, the toxin might be starting its activity from the very early stage. Mateur (1957) reported a toxin from germinating urodospores of race 15-B of Puccinia graminis var. tritici. The inhibition of seed germination particularly of bajra can be used as a bioassay test for Sg-toxin after further standardisation. The inhibition of seed germination might be due to the effect of toxin on the vital parts of the seed. However, the histochemical studies of the toxin treated seeds did not show any structural damage. The changes might be in ultrastructures or in the physiology of germinating seeds. This requires further study.

The results of radicle length of germinating seeds (Table 2) showed a varied trend. Increased length of the radicle was noticed in all the tests especially in bajra (Plate 2). It is known that S.graminicola affects mainly leaves and shoot portion of the plants. The increased radicle length might be due to some growth promoting substances in Sg-toxin whose effect was confined to the radicle. It needs further confirmation and studies for concluding the nature of the substances involved. Bajra plants infected by S.graminicola in later stage produce the green earhead where ovaries will be converted into leaf like structures. This also suggests the association of growth promoting substances with downy mildew. The growth regulators were reported to be involved in plant diseases caused by bacteria and fungi (Thimann 1966 and Wani et al., 1977). In general, toxins from phytopathogenic fungi are reported to inhibit the radicle length of germinating seeds (Guensi and McCalla, 1967, and Scheffer and Ullstrup, 1965). However, Kuo and Scheffer (1967) and Kuo et al.(1969) reported that dilute solution of Helminthosporium carbonum toxin stimulated root growth of corn seedlings. The reasons for varied response of radicle lengths to Sg-toxins are not known.

The plumule length was adversely affected by Sg-toxin treatment in all the cases except in blackgram and navane (Table 2). These results suggested that Sg-toxin affected someof the activities of growing shoot tissues resulting in reduced plumule length. Downy mildew affected plants are generally stunted. The reduced plumule length supported and clarified the reason for the stunted growth of such infected bajra plants. The

pathogen also affects the shoot portion of the plant, but the exact mechanism of reduced plumule length could not be understood even by microscopic histochemical studies. But it may be affecting ultra-structures of the seeds or metabolic processes which may be detected by electron microscopic studies. With further standardization, this method may serve as one of the methods for bioassay of Sg-toxin.

The studies on host specificity of Sg-toxin indicated that it was not a specific toxin. Rai (1977b) reported that Plasmopara viticola toxin was non-specific. It was noted that Sg-toxin did not have effect on maize, paddy and green gram plants. Safeeulla (1976) reported that S.graminicola did not infect maize in India. He proposed that maize varieties in India may be resistant to this pathogen or the fungus species prevalent in India belongs to a different pathogenic race. The results of the present studies suggested that maize and paddy were quite resistant to Sg-toxin. From the non-specific nature of the Sg-toxin, it can be considered that the specificity of S.graminicola in nature might be due to its nutritional requirements. If a non-host plant could provide the nutrients required by the fungus for its growth, then it may attack the non-host plant also. For example, ragi, blackgram, tomato, wheat, etc., could be attacked by S.graminicola if its nutritional requirements are met. These studies revealed that the mechanisms of host-specificity in case of S.graminicola might be based on the nutritional hypothesis, and these results are of much biological importance. Safeeulla (1976) had successfully grown S.graminicola on ragi callus which

was not a host for the pathogen. Even after removing the callus from medium, the fungus survived for some time in the absence of callus on the medium.

The Sg-toxin did not affect the growth of any of the microorganisms tested (Table 5). It suggested that Sg-toxin was group specific if not host-specific. It was toxic to some plants and not at all to micro-organisms. Braun (1955) used Chlorella vulgaris as bioassay organism for the activity of the Wildfire toxin and noted that toxin inhibited completely the growth of this alga. Ikawa et al.(1969) suggested the use of Chlorella in mycotoxin and phycotoxin research. Sullivan and Ikawa (1972) found wide variations in the inhibition of growth of four strains of Chlorella pyrenoidosa and a strain of Chlorella vulgaris by some of the mycotoxins.

The bajra plants grown from seeds, soaked in Sg-toxin almost mimicked the symptoms of downy mildew disease. These results indicated the possible role of Sg-toxin in the causation of most of the downy mildew symptoms like chlorosis, curling of leaves and wilting of the plants. The inhibition of seed germination was 40 per cent in green house condition and 27 per cent in laboratory condition when treated with 2 per cent toxin. The symptoms in toxin treated plants, appeared in stages which indicated the step wise action of Sg-toxin on bajra leaves. In first stage chlorosis was initiated and in second stage, it was followed by yellowing

and curling from tip towards leaf base and at final stage the leaves wilted completely. All these results clearly indicated that Sg-toxin affected the metabolic activities of the plant and these results were partially supported by the histochemical studies.

The histochemical studies of leaves revealed that during I stage of symptoms development there were no significant changes in periodic acid Schiff's test positive granules in the cells of the epidermis and mesophylls. But the increased number of polysaccharide grains was noticed in bundle sheath cells (Plate 4). This increased number of polysaccharide granules in bundle sheath cells of leaves might be due to either an increased activity of the polysaccharide synthesising enzymes or reduced activity of the enzymes which are responsible for the incorporation of the polysaccharides in to different cell components. During II and III stage, the polysaccharide granules in bundle sheath cells were lessened and then completely disappeared which indicated that polysaccharide granules were subsequently broken down and synthesis of polysaccharide also might have been stopped. It has been reported earlier that commonly starch accumulates in plants infected by obligate parasites. Generally, it accumulates in granules in chloro-plasts of photosynthetic tissues (Akai et al., 1967) and in host cytoplasm in non-photosynthetic tissues (Williams et al., 1968). Mirocha and Zaki (1966) reported that starch decreased at infection sites of rusted bean leaves soon after infection, increased sharply just before sporulation, and then decreased sharply after sporulation. Macdonald and Strobel (1970)

reported that the starch content of rust infected wheat leaves decreased from 5 to 9 days, increased from 9 to 12 days to double that of healthy leaves, and decreased from 12 to 15 days after inoculation. Tanaka and Akai (1960) have hypothesized that increased starch content in rice leaves infected with Cochliobolus miyabeanus (Ito and Kuribay) Dickson was due to a decrease in beta-amylase activity. Keen and Williams (1969) found increased specific activities of the starch synthesizing enzymes, UDP-glucose pyrophosphorylase and starch synthetase, during starch accumulation in cabbage hypocotyls infected with Plasmodiophora brassicae.

The results of methyl green pyronin test for DNA and RNA showed that upto II stage there were no significant changes in DNA and RNA contents but in III stage the nuclei of most of the cells disappeared and also the plastids. Most of the DNA and RNA was found degraded. This indicated that the Sg-toxin affected polysaccharides in the beginning and only during the later stages the DNA and RNA were degraded. It clarified the mechanism of sequential symptom causation in the toxin treated plants.

The results of test for protein indicated that during earlier stages of symptoms development the protein particles became less stainable and the shape also became distorted, indicated the degradation of plastid material. During II stage particles became still smaller and some of the bundle sheath cells lost protein bodies completely. In III stage the protein positive bodies found completely disappeared, indicating the successive degradation of those particles due to the toxin activity(Plate 5).

All these results supported the findings of earlier experiments wherein the stage wise symptoms were noticed in the toxin treated plants. Lewis and Goodman (1962) reported the loss of integrity of foliar tissues primarily in the spongy parenchyma soon after exposure to colletotin. Luke et al.(1966) reported several changes in oat root cap tissues, first a dark staining material appeared between the plasma membrane and the cell wall, followed by partial separation of the plasma membrane from the cell wall, and disruption of the internal membrane systems. Park et al. (1976) found that due to A.kikuchiana toxin the first change in ultra-structure was invagination of plasma membrane. They reported that the plasma membrane was the site of initial effect of A.kukuchiana toxin.

In case of germinating seeds no significant changes could be observed due to Sg-toxin treatment. However, the possibility of damage to ultra-structures and at lower level cannot be ruled out. Such changes if any may be detected by electron microscopy.

The experiments related to inactivation of Sg-toxin revealed that dried Sg-toxin was quite a stable compound over extended period of time. It can be stored in desiccator or in refrigerator for 18 months without loss of its activity. Rai (1977b) reported that toxin produced by Plasmopara viticola was quite stable. Unlikely, activity of H.maydis toxin was reduced by 50 per cent in 24 hr. at 25 C (Comstock, 1971). The Sg-toxin was quite stable to temperature also. It was stable up to

95 C but inactivated at higher temperature. Similarly, H.sacchari toxin was stable over extended periods of time and heat, up to 144 C (Steiner and Byther, 1971). The partial acid hydrolysis of Sg-toxin by 1 N H_2SO_4 indicated that exposure of toxin for 15 and 20 min reduced its activity and above 20 min its activity was lost completely (Fig.5). Such loss in activity of Sg-toxin due to 1 N H_2SO_4 might be due to loss of critical sugar or amino acid residues from the parent molecule resulting in loss of biological activity. Similarly, about 50 per cent loss in activity of toxic polysaccharide produced by Corynebacterium sepedonicum was reported by Strobel (1967) when refluxed for 1 min in 0.5 N H_2SO_4. These results suggested that there is no possibility of inactivation of Sg-toxin in host plant in nature either by temperature, acidity or storage over long period of time.

The Sg-toxin was immunogenic and the antiserum with 1 : 1280 titer was obtained against the toxin. The Sg-crude toxin and its fractions-I and II formed clear bands of identity with the homologus antiserum showing that they were serologically identical (Ouchterlony, 1958, and Rai and Strobel, 1969). The immunogenic nature of the toxin might help in the detection of the disease in host plants in an early stage of the disease and the method is quicker and economical.

Purification studies of the Sg-toxin by following Sephadex G-200 column chromatography revealed that it consisted of two fractions (Fig.6)

and fraction-II was recovered in more proportion (51 per cent) than fraction-I (27 per cent). The estimated molecular weights of both these fractions were 2,73,200 and 61,630 respectively. The toxin produced by Plasmopara viticola was made up of only one fraction having molecular weight of 89,760 (Rai, 1977b).

The intrinsic viscosity of the Sg-crude toxin was 0.122 decilitre/g and that of fractions-I and II was 0.089 and 0.047 decilitre/g respectively. The intrinsic viscosity values increased with increasing molecular weight of the compounds (Table 5).

The Sg-crude toxin and its fraction-II had pH near neutrality, but fraction-I had slightly acidic pH (6.70). The electrophoretic studies revealed that Sg-toxin was cationic in nature.

The results of recovery of anionic, cationic and neutral fractions from ion-exchange chromatography indicated that an anionic and neutral fractions dominated in crude toxin whereas in fractions-I and II cationic fraction dominated. This might be due to more loss of sugar and sugar acid residues during the process of gel chromatography, acid hydrolysis and ion-exchange chromatography.

The analyses of different fractions of Sg-crude toxin and its fractions revealed that they are made up of various sugars, sugar

acids (Table 6) and amino acids (Table 7). These results suggested that Sg-crude toxin and its purified fractions were glycopeptide in nature. Similarly, Rai (1977b) reported that Plasmopara viticola toxin was glycopeptide in nature and made up of sugars, sugar acids and amino acids. The empirical formulae of Sg-crude toxin and fraction-II were calculated as $C_7H_{12}O_{13}N$ and $C_8H_{15}O_{32}N$ respectively.

SUMMARY

VI. SUMMARY

Bajra plants (10 days old, 6-7 cm tall) of HB-3 variety were inoculated with sporangiospores of Sclerospora graminicola. The sporangial inoculation method was proved superior over oospore inoculation. The pathogen was maintained on bajra plants by growing in sick soil.

The toxic compound was isolated from powdered downy mildew infected bajra plants but such toxic compound could not be isolated from healthy plants. The Sg-toxin was present in infected bajra plants in a very low quantity (0.495 per cent). The plant cuttings of bajra and tomato were used for bioassay of toxicity of Sg-toxin and tomato plant was found to be more sensitive than bajra plant. There was an inverse relationship between toxin concentration and time required for wilting upto 0.125 per cent level. The purified fraction-II of Sg-toxin was more toxic than fraction-I and Sg-crude toxin. The dilution end point of toxicity of fraction-II was 0.03 per cent whereas that of fraction-I and Sg-crude toxin was 0.12 per cent and 0.06 per cent respectively.

The Sg-toxin inhibited the germination of seeds in general, however, more conspicuous results were recorded in case of bajra and ragi seeds. The germination of bajra and ragi seeds was inhibited by 27 per cent and 12 per cent respectively due to 2 per cent toxin treatment of seeds. In

general, the radicle length of germinating seeds was increased particularly of bajra and plumule length was decreased in all the cases except black gram and mavane.

The host specificity studies revealed that Sg-toxin was a non-specific toxin however paddy, green gram and maize plants were not affected by the Sg-toxin.

Bajra plants grown from the seeds soaked in Sg-toxin mimicked all the symptoms as that of S.graminicola except downy growth. The Sg-toxin did not affect growth of any of the microorganisms and it suggested that Sg-toxin was group specific if not host-specific toxin.

There were no significant morphological and histochemical changes in radicle, coleoptile or embryo of toxin treated and control seeds. Significant histochemical changes were noted in leaves from the plants grown from seeds soaked in Sg-toxin. In the I stage of symptoms development polysaccharide grains in bundle sheath cells were increased and then subsequently decreased during II stage and completely disappeared in III stage. The DNA and RNA contents were not affected during I stage however, at III stage most of the DNA and RNA was completely degraded. During subsequent stages of symptoms development proteins were degraded and plastids were found to be degraded.

The Sg-toxin was antigenic in nature and the crude toxin and its fractions-I and II were serologically related.

The Sg-toxin was quite stable compound and could be stored in dried form in desiccator or refrigerator for longer time. It was heat resistant and was active even when exposed to 95 C for 10 min. The partial hydrolysis of Sg-toxin in 1 $\underline{N}$ H_2SO_4 reduced its biological activity.

It was dark brown and crystaline in nature. The Sg-toxin was separated into two fractions by Sephadex G-200 gel chromatography. The fraction-II was recovered in more percentage than fraction-I from column. The fraction-I had molecular weight of 2,73,200 and the fraction-IIhad 61,630. The intrinsic viscosity of the Sg-crude toxin was 0.122 deciliter/g and that of fractions-I and II was 0.089 and 0.047 decilitre/g respectively.

The Sg-crude toxin had pH of 6.98 and the fractions-I and II had pH of 6.70 and 6.90 respectively. The electrophoretic studies indicated that Sg-toxin was cationic in nature.

The acid hydrolysed Sg-crude toxin and its fractions-I and II were fractionated into an anionic, cationic and neutral fractions. The Sg-crude toxin contained arabinose, glucose and two unknown sugars, and

the fractions-I and II contained arabinose, glucose and one unknown sugar in each fraction. The Sg-crude toxin contained D-galacturonic acid and three unknown sugar-acids, whereas fractions-I and II contained three and two unknown sugar acids respectively. In all Sg-crude toxin contained 24 amino acids and fractions-I and II contained 21 and 22 amino acids respectively. The empirical formulae of Sg-crude toxin and its purified fraction-II were $C_7H_{12}O_{13}N$ and $C_8H_{15}O_{32}N$ respectively.

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* Original not seen