EFFECT OF THE PRIMARY DETERMINANT OF PATHOGENICITY FROM PERICONIA CIRCINATA ON SORGHUM VULGARE

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This is to certify that the

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ABSTRACT

EFFECT OF THE PRIMARY DETERMINANT OF PATHOGENICITY FROM *PERICONIA CIRCINATA* ON *SORGHUM VULGARE*

by Isis S. Mansour

Periconia circinata produces a substance in culture that is toxic to susceptible grain sorghum, but is harmless to other non-host plants. Differential effects of this toxin on susceptible and resistant sorghum plants were studied. Effects were compared with known effects of *Helminthosporium victoriae* and *H. carbonum* toxins on their corresponding hosts (oats and corn).

A general idea of the kinetics of toxicity was obtained by using a series of different concentrations, temperatures, and exposure times. The percent of seedlings that failed to grow roots varied directly with toxin concentration and exposure time. The expression of toxicity by susceptible seedlings was increased as the temperature was raised from 5 to 21 to 32°C.

Resistant and susceptible cuttings were allowed to take up measured amounts of toxin in an effort to determine the nature of resistance. A significant amount of toxin was recovered from both susceptible and resistant toxin-treated cuttings, always with a higher percent recovery from resistant than from susceptible tissue.

Therefore, resistance does not depend on superior ability of the resistant cell to inactivate toxin. Toxin appears to be adsorbed or inactivated to some extent by both resistant and susceptible tissues. It is possible that resistant cells lack or have fewer toxin receptor sites than do susceptible cells. Susceptibility or resistance to PC-toxin appear to be based on constitutive factors.

Toxin caused an increase in respiration of intact susceptible root and leaf tissues. It had no effect on oxidation or phosphorylation by isolated mitochondria from susceptible seedlings. Apparently the increase in oxygen uptake in the intact tissue is a secondary effect of toxin. A system or systems other than the enzymatic reactions of the Krebs cycle could be the primary site affected by toxin, which in turn could lead to increased respiration.

Another effect of PC-toxin was to decrease incorporation of labeled amino acids into the TCA-precipitable fraction of susceptible leaves. There was no such effect on resistant tissue. Toxin also, decreased incorporation of uridine into the RNA fraction of susceptible leaves. Again there was no effect of toxin on uridine incorporation into the RNA fraction by resistant leaves. Toxin had no effect on RNase activity in susceptible tissue as determined by tissue extracts at pH 5.

A marked reduction in active uptake and retention of C^{14}-amino acids was observed when susceptible roots were treated with PC-toxin. Resistant roots were not affected. It appears likely that PC-toxin affects sites on the cell surface that are responsible for active transport across the membranes. A breakdown in transport of compounds to synthetic sites could explain the inhibitory effect of toxin on incorporation of amino acids and uridine into protein and RNA, respectively.

Toxin increased electrolyte loss from susceptible treated tissues, but not from resistant ones. This phenomenon generally reflects change in permeability of cell membranes which consequently lead to disturbance and alteration in protoplasmic compartmentalization. Such changes could lead to many physiological and metabolic changes in infected tissues.

P. circinata was found by histological techniques to invade susceptible sorghum root tissue in a way similar to many other plant pathogens. The fungus is clearly a root invading organism with a slow growth rate in its host.

The several lines of evidence indicate that the action of PC-toxin on susceptible sorghum cells parallels the effects of HV-toxin on susceptible oat cells. However, PC-toxin is much slower and less dramatic in its action than is HV-toxin. Experiments with cell wall free

protoplasts have been very useful in elucidating the action of HV-toxin. Such experiments with PC-toxin have not been conclusive, because of fragility of the preparation and a delayed effect of the toxin.

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By

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INTRODUCTION

We now have clear evidence that specific compounds produced by certain plant pathogens are involved in disease development (34). Some of these substances appear to be essential for pathogenicity, and have been called "primary determinants." It is equally clear that some pathogenic microorganisms produce substances that are involved in disease development, but that are not necessary for pathogenicity. Such substances have been called "secondary determinants" (47). My work is concerned with a primary determinant or toxin produced by Periconia circinata (Mangin) Sacc., a fungus pathogen of grain sorghum (Sorghum vulgare var. subglabrescens).

Primary or essential determinants are known from at least 5 different plant-infecting fungi: Helminthosporium victoriae, Meehan and Murphy, H. carbonum Ullstrup, Periconia circinata, Alternaria kikuchiana, Tanaka, and some strains of A. mali (47). The evidence for a causal role of these compounds in disease development rests mainly on work with H. victoriae toxin (HV-toxin). All pathogenic strains of H. victoriae produce toxin in culture; strains which lose the ability to produce toxin

are non-pathogenic to Victoria-type oats (48). The substance is toxic only to the host which is susceptible to the fungus. Nishimura and Scheffer (28) postulated that HV-toxin is involved in initial establishment of the fungus in the host, because the spores release toxin on germination. All known biochemical changes caused by H. victoriae infection are also induced by HV-toxin (47). Recent evidences indicate that the plasma membrane is the primary site of action of the HV-toxin (42) in disease development.

P. circinata toxin (PC-toxin) is toxic only to certain cultivars of grain sorghum or milo and is harmless to other sorghum cultivars as well as to non-hosts. The toxin produces all the disease symptoms caused by the fungus, and confers pathogenicity to the organism (44). PC-toxin is somewhat more stable than HV-toxin in slightly alkaline medium, and can be evaporated to dryness (34, 44). It is thus much easier to concentrate and manipulate PC-toxin than HV-toxin for experimental work. There is much previous physiological information on H. victoriae toxin (34, 47), so it was taken as a model for further work with PC-toxin. Some physiological effects of PC-toxin on both susceptible and resistant sorghum plants were examined. My studies include uptake of PC-toxin by sorghum seedlings, recovery of toxin from treated resistant and susceptible cuttings, and effect of toxin on protein and RNA synthesis. Further studies were on loss of electrolytes from treated

tissues, effect of toxin on mitochondrial reactions, and responses of cell wall free protoplasts to toxin. The only cellular response previously reported for PC-toxin is the increase in oxygen uptake by the treated susceptible plant (47).

It is of great importance to learn how the known pathogen-produced determinants affect sensitive plants, and to determine whether or not a generalization of their action can be made. Such highly selective toxic compounds are thought to be useful models for studying the biochemical and physiological mechanisms of host-parasite interaction. They make it possible to study the metabolism of a diseased plant, at the same time excluding many complexities of two living organisms and their interactions. Recognition and isolation of pathogen-produced determinants of disease could lead to an understanding of disease resistance.

LITERATURE REVIEW

All the background literature on *P. circinata* toxin will be reviewed below. Those parts of the literature on *H. victoriae* toxin pertinent to this work will also be covered, since much of my work was based on this model. Evidences for the involvement of the host-specific toxins in disease development, the criteria of choosing these factors as primary determinants, and their physiological and biochemical effects on sensitive plants were considered in recent reviews (34, 47, 60). There was no evidence for a chemical basis of pathogenicity until the host-specific toxins were discovered (34).

Milo disease was first known in the southwestern states of the United States in 1924. The name crown and root rot of milo was first given to the disease in 1932 (9). Undetermined soil pathogens were considered to be the causal agents (9), but in 1937 the disease was attributed to *Pythium arrhenamanes* (10). This is surprising, in retrospect, because sorghum varieties resistant and susceptible to the milo disease were affected equally by *P. arrhenamanes* (22). Leukel in 1948 described the causal agent of milo disease as *Periconia circinata* (Mangin)

Sacc. This fungus had the proper host selectivity, affecting only certain cultivars of Sorghum vulgare var. subglabrescens, while other cultivars were not affected. The same species was first described by Mangin in France in 1899 as a saprophyte from wheat plants affected with foot rot (22, 34). The earlier confusion about the organism causing milo disease can be attributed to the uncertain growth characteristics of P. circinata in culture. It grows very slowly in culture and is usually overrun by rapidly growing saprophytes occurring in the infection sites (22, 34). P. circinata is a soil-borne fungus which invades the roots and the lower internodes of the culm (34). Flentje (13) and Walker (56) stated that P. circinata was not an invading fungus but killed susceptible sorghum plants by toxic action. No evidence was cited for this assumption.

Leukel (22) suggested and provided indirect evidence that toxin having the same specificity as the fungus might be involved in the milo disease. He planted susceptible and resistant sorghum varieties in pans heavily inoculated with P. circinata. Two weeks later all plants of the susceptible varieties were dead. All plants were removed and the pans were steamed at 100°C for 1 hour, after which they were planted again with susceptible and resistant sorghum seeds. Again the susceptible seedlings died while the resistant seedlings were not affected, suggesting the

presence of a thermostable host-selective toxin. This was further investigated by Scheffer and Pringle (44), who proved that the fungus produced a host-specific toxin. All P. circinata strains tested which were pathogenic to sorghum cv. Colby and to several other susceptible cultivars produced a substance in culture filtrates toxic to these plants but harmless to sorghum cultivars known to be resistant to the fungus. Non-host plants were not affected by the toxic substance. Loss of pathogenicity was correlated with loss of toxin production by the fungus (44).

At least 2 related host-specific toxic compounds were found from P. circinata. These were separated by ion exchange column chromatography and countercurrent distribution (33, 36); they were designated as Periconia toxins A and B, both of which yielded amino acids on hydrolysis. Originally, only toxin A was found in cultures, and it was produced consistently throughout the experiments. Later, toxin B appeared in culture filtrates and reached higher and higher concentrations. Eventually, production of toxin B fell until it could no longer be found in culture filtrates (36).

Periconia toxin A was crystallized and purified (33, 35). The purity of the crystalline material was established by countercurrent distribution, and by ion-exchange and paper chromatography. The toxin is a low molecular weight polypeptide composed of 6 moles alanine,

4 moles aspartic acid, 2 moles each of glutamic acid and serine (35). Molecular seiving experiments showed that the molecular weight is less than 2000 (35). The crystalline material retained its host specificity and inhibited susceptible root growth in solutions at concentration of 0.1-0.01 µg/ml. The stability of the compound decreased with purification. Factors responsible for stability of the toxin *in vivo* or in crude preparations have not been identified (35).

Symptoms induced by PC-toxin are the same as those induced by the fungus. There is evidence that susceptibility to the disease and to the primary determinant has the same genetic basis (47).

The model for PC-toxin studies was *Helminthosporium victoriae* toxin, which will now be considered. *H. victoriae* toxin was discovered by Meehan and Murphy (26) as a factor in development of Victoria blight of oats. This work was later confirmed by Luke and Wheeler (24). HV-toxin is a low molecular weight (800-2000) compound containing a cyclic secondary amine ($C_{17}H_{29}NO$) known as "victoxinine," and a peptide made up of aspartic acid, glutamic acid, glycine, valine, and leucine. It is more labile than PC-toxin, being degraded by mild alkali to victoxinine and the peptide (34, 47). HV-toxin has a higher biological activity than any other known primary determinant. A concentration of 0.0002 µg/ml of HV-toxin (47) will give

complete inhibition of susceptible oat seedling roots, whereas for PC-toxin a concentration of 0.1 µg/ml is needed to give complete inhibition of susceptible sorghum roots. Uptake studies with HV-toxin by susceptible oat seedlings indicated that uptake was a simple physical process not affected by temperature over a wide range (5-37°C), by various metabolic inhibitors, or by a wide range of osmotic concentrations in assay solutions (46). The toxicity of HV-toxin was decreased by breakdown products of the toxin or by bisulfite in the assay solution. These and other circumstantial evidences led Scheffer and Pringle (46) to suggest receptor sites for the toxin in susceptible cells which are different or lacking in resistant cells.

An opposing view is that of Romanko (38), who reported up to 40% recovery of the activity when toxin was taken up by susceptible oat cuttings; there was no recovery from resistant treated cuttings. Therefore it was suggested that the nature of resistance in oats depends on the efficiency of toxin inactivation by tissue. Furthermore, the ability of plants to inactivate toxin was said to be a property specific to intact, living cells, because extracts and homogenates of resistant plants did not inactivate toxin (60). These results on toxin recovery from treated tissues could not be confirmed by Scheffer and Pringle (46).

The increased rate of respiration that characteristically accompanies infection of plant tissues by many pathogens has been the subject of much inquiry (3, 27, 54). The factors responsible for such increase have not been identified, except for the toxin involved in H. victoriae infection. Several mechanisms have been reported to account for the increased rate of respiration; these explanations include increased activity of normal metabolic pathways, shifts to new pathways, activation of specific enzymes, and uncoupling of oxidative phosphorylation (54). Similarly, the mechanism of increased respiration induced by host-specific toxins remains undefined. Allen (2) suggested that toxins might act as uncoupling agents and hence stimulate respiration . Reduced sensitivity of HV-toxin treated tissues and tissue infected with H. victoriae to 2, 4-dinitrophenol (DNP) was given as proof of uncoupling (19). However, decreased sensitivity to DNP could result from other changes such as increased utilization of ATP by synthetic reactions (21, 60). Unlike HV-toxin, DNP is non-specific, and its effect on respiration is quantitatively much less than is the toxin (38).

Studies with isolated mitochondria from susceptible oat seedlings indicated no effect of the toxin on succinoxidase activity (45). Apparently mechanisms other than those involved in Krebs cycle reactions are of primary importance. This, in turn, could affect respiration in

various ways. Other workers (16, 19) indicated that activation of terminal oxidases other than cytochrome oxidase might lead to higher oxygen uptake by toxin treated tissues. Wheeler and Black (58) suggested that permeability changes in mitochondrial membranes induced by HV-toxin might be responsible for respiratory increase. They were nevertheless unable to detect effects on mitochondrial membranes (7).

Some effects of HV-toxin on nitrogen metabolism parallel the effects reported for several plant diseases (47). There are, however, conflicting evidences for changes in plant proteins after infection by fungi and bacteria. There is good evidence for increased protein around certain infection sites (6), but it is by no means certain that any new kinds of host proteins are involved. The new antigens in host cells adjacent to black rotted sweet potato tissue (55) could be the result of breakdown products of host tissue or of diffusable fungal products. The best case of altered host protein after infection comes from work of Reddi, who reported qualitatively different ribonuclease in crown gall and normal cells (37). There are reports of infection-induced changes in the functional properties of existing protein; for example, a decrease in photosynthetic CO_2 fixing capacity of chloroplast fraction 1-protein after infection of rice leaves with Pericularia oryzae (1). The nature of this change is

uncertain and no definite cause and effect relationships in disease development have been demonstrated. Also, the role of such changes in defense reactions of host plants is yet to be determined.

There are indications of decreased protein levels after infection by some microorganisms. Wildfire toxin, a secondary determinant produced by Pseudomonas tabaci, increased protein degradation (11), resulting in accumulation of free amino acids in tobacco tissue. This was found to be associated with a decrease in RNA level in toxin treated tissues (23). Decrease in RNA level was thought to be correlated with a higher RNase activity in toxin treated tobacco leaves than in the controls. The effect of wildfire toxin on RNA metabolism was counteracted by 10^{-4} M kinetin, but this treatment did not affect RNase activity (23). These results do not explain the effect of wildfire toxin on protein metabolism.

An early effect of HV-toxin on susceptible tissues is the inhibition of incorporation of C^{14}-amino acids and uridine by tissues into a TCA-insoluble fraction. No such effects were found in resistant tissues (47). These findings do not necessarily mean that HV-toxin affects protein synthesis directly. Other evidence indicated that HV-toxin causes a disturbance in uptake and transport mechanisms, preventing movement of these compounds

to the sites of protein and RNA synthesis. HV-toxin did not inhibit protein synthesis when added to ribosomes from reticulocyte cells which are affected by all known inhibitors of protein synthesis (41).

Alterations in cellular permeability are well-known results of infection in plants. Thatcher (50,51) in his studies on osmotic and permeability relations in parasitism, showed that several infections caused an increase in permeability of the plasma membranes of susceptible cells in the vicinity of the parasitizing mycelium. He ascribed increase in permeability to derangements in the plasma membrane. An obvious and very rapid effect of HV-toxin is the leakage of solutes from tissues (59). Leachates were reported to include potassium, amino acids, and other nitrogenous compounds (7). The rate of loss of electrolytes has a low temperature coefficient, and the rate is not affected by oxygen tension. These results indicated to Black and Wheeler (58) that HV-toxin affects the balance of salts and other materials within the cells. This effect on permeability might indirectly affect respiration rates (58). Amador and Wheeler (4) found that susceptible tissue pretreated with HV-toxin and leached in distilled water had a much lower rate of respiration than similar tissue not leached. Derangement in the cell membrane is an obvious effect of HV-toxin (47), but the site of action or how it affects the plasma membrane is not clear.

Other cellular phenomena which could reflect changes in permeability of plasma membranes were studied (42). Plasmolytic ability of toxin treated susceptible cells was lost after a short time of treatment. Resistant tissues were not affected in this way. HV-toxin also stopped membrane-regulated active uptake of exogenous amino acids and inorganic phosphate after a brief exposure. Apparent free space increased in susceptible but not in resistant oat roots after toxin treatment. All these effects could be attributed to membrane damage.

The effects of HV-toxin on free protoplasts from oat coleoptiles also indicated a membrane lesion. Protoplasts from susceptible oat coleoptiles burst within one hour after toxin treatment, whereas protoplasts from resistant coleoptiles were not affected (41, 42). Cyclosis or protoplasmic streaming stopped quickly in susceptible protoplasts after toxin treatment. Electron microscopic evidence of toxin-induced disruption of the plasma membrane of susceptible cells was also reported (25), but such gross lesions were apparent only in tissues exposed to toxin for a long time.

MATERIALS AND METHODS

Plants and bioassay.--Sorghum cultivars susceptible (cv. Colby) and resistant (cv. RS-610) to Periconia circinata and to its toxin were used. Seeds were germinated on moist filter paper in petri dishes at 22-24°C. In some cases seedlings were grown in vermiculite in glazed pans, supplied with nutrient solution or water at 22-24°C. Larger plants for some experiments were grown in the greenhouse in peat-soil-sand mixture. Cuttings were taken from 15-22 day old plants with 3-5 fully expanded leaves.

Inhibition of seedling root growth was used as a standard bioassay for toxin (32). Seeds were germinated between sheets of moist filter papers at 30°C for 24 hours. Serial dilutions of solutions containing toxin were made with distilled water. Five ml of each dilution was placed in 60 x 15 mm petri dishes containing 5 germinated seeds. The assay end point was determined after 4 days of incubation as the highest dilution which limited susceptible root growth to 1.0 cm or less. Susceptible seedlings in distilled water as well as resistant seedlings in toxin

solutions were used as controls. Control roots grew from 3-10 cm during the assay period.

Toxin preparation.--A highly virulent strain of Periconia circinata was grown for approximately 21 days on a modified Fries No. 3 basal medium supplemented with 0.1% Difco yeast extract (33). The culture filtrate was concentrated in vacuo, precipitated with methanol, and adsorbed on Norit A-Celite in a large column, as described previously (33). Toxin was released from the Norit with a 10% pyridine solution. After removal of the pyridine, the preparation was stored at 5°C as a stock solution. For further purification, one ml of the Norit eluate was placed on a 25 x 1.5 cm Bio-Gel P-2 column. The column was prepared, washed and developed with distilled water. The effluent was collected in 10 ml fractions. A portion of each was serially diluted with water and assayed as mentioned above, while a 1 ml portion was used to determine dry weight. These measurements were done to develop an activity elution profile (Fig. 1). Fractions 2, 3, and 4 showed high activity as measured by bioassay. Maximum activity was found in fraction 3, which gave complete inhibition of susceptible sorghum roots at 0.11 µg per ml. This preparation was twice as active as the eluate from Norit-Celite column, which gave complete inhibition of root growth at 0.225 µg

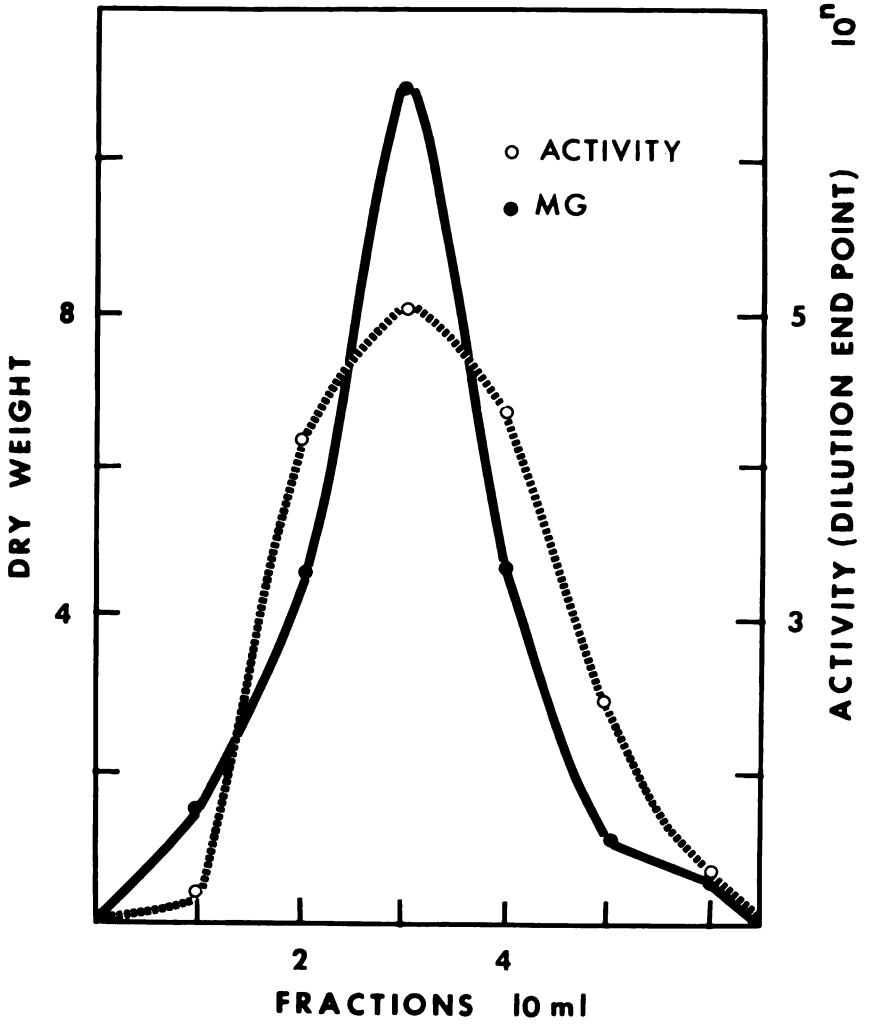

Figure 1.--Elution profile for PC-toxin from Bio-Gel P-2 column (25 x 1.5). The column was loaded with 1 ml of eluate from Norit-Celite column, and developed with distilled water. Dry wt. is in mg/ml.

per ml. The Bio-Gel preparation was used in all experiments, unless indicated otherwise.

Preparation of mitochondria.--The extraction medium used by Wu and Scheffer (62) was slightly modified by substituting Tricine [N-tris (hydroxymethyl) methylglycine] (14) for tris (hydroxymethyl) amino methane. The medium contained the following: sucrose, 0.4 M; Tricine, 0.2 M; ethylene-diamine tetra acetic acid (EDTA), 0.005 M; dibasic potassium phosphate, 0.01 M; and sodium citrate, 0.02 M. The solution was adjusted to pH 7.9.

Tricine was also substituted for tris buffer in the washing medium, which contained the following: sucrose, 0.2 M; Tricine, 0.1 M; EDTA, 0.0025 M; dibasic potassium phosphate, 0.005 M; and sodium citrate, 0.005 M. The pH was adjusted to 7.3.

Mitochondria were isolated from etiolated sorghum seedlings using the method of Wu and Scheffer (62) with slight modification. Etiolated sorghum seedlings were grown for 7-10 days on porcelain plates, covered with cheesecloth and moistened with White's nutrient solution. Seedlings were cut into pieces, divided into samples of 30 gm each, and chilled at 3-4°C for one hour. All preparation was done in the cold room at about 3-4°C using prechilled glassware and solutions. The tissues were then vigorously ground in a mortar for 2-3 minutes with half

their weight of acid-washed sea sand and with a volume of extraction medium twice the weight of the tissue (ml=gm). The homogenate was filtered through six layers of cheesecloth, centrifuged for 10 minutes at 1,000 X g and the pellet was discarded. The supernatant solution was recentrifuged for 30 minutes at 15,000 X g and the pellets containing the mitochondria were suspended in washing medium equal in volume to the original weight of the sample. After centrifugation for another 30 minutes at 15,000 X g, the supernatant fluid was discarded and the final washed pellet was suspended in 2-3 ml of 0.25 M sucrose solution.

Oxygen uptake measurements.--For experiments on O_2 uptake by tissue, 100-250 mg (green weight) tissue samples were placed in Warburg flasks with or without toxin at pH 5.4. In some experiments tissue samples were pretreated with toxin, washed, then placed in the flasks containing 2 ml phosphate buffer (pH 5.4). All tests were made at 30°C with duplicate flasks for each treatment. The equilibration time was 10 minutes. Readings were taken at 10 minute intervals for 1 hour or more. Results were calculated as oxygen uptake in µl/flask.

Oxygen uptake by mitochondria was also determined manometrically. Mitochondrial suspension (0.4 ml) was placed in each flask to give a total volume of 2.2 ml

reaction mixture. The pH of reaction mixture in all cases was 7.3 and the temperature was 30°C. The reaction mixture contained the following, per flask: substrates, (potassium salts of succinate or α-ketoglutarate) 0.025 M; sucrose, 0.2 M; monobasic potassium phosphate, 0.07 M; magnesium sulphate, 0.0075 M; adenosine triphosphate (ATP), 0.001 M; cytochrome c, 0.1 mM; nicotinamide adenine dinucleotide (NAD), 0.13 mM; thiamine pyrophosphate (TPP), 0.13 mM; coenzyme A (CoA), 0.013 mM; and malonate, 0.025 M. Equilibration time was 5 minutes, and readings were taken at 10 minute intervals. For P:O ratio determinations, 0.2 ml hexokinase solution (2 mg/ml type IV in 0.5 M glucose) was added to the flask side arm and tipped into the reaction mixture after equilibration. Activity was expressed as oxygen uptake (µl) per flask.

Phosphorylation by mitochondria.--Inorganic phosphate was determined by the method of Fiske and Subbarow (12). Oxygen uptake was measured as above for 40 minutes after which 2 ml of 10% chilled trichloroacetic acid (TCA) was added to the reaction mixture in each Warburg flask, and a 2 ml sample of the resulting mixture was removed for P_i determination. Samples were centrifuged at 15,000 X g for 15 minutes at 4°C; 1 ml of the supernatant solution was diluted with 9 ml chilled glass distilled water. From this

dilution 0.2 ml samples were removed and added to 8.4 ml water to which was added 1 ml 2.5% ammonium molybdate and 0.4 ml 0.25% aminonaphtholsulfonic acid. The color that developed after 15 minutes incubation was measured at 660 mμ, using a Klett-Summerson colorimeter with filter no. 66. Separate flasks were used to determine phosphate at the beginning of the experiment. After 5 minutes equilibration in the Warburg water bath, TCA was added to the mixture before hexokinase was tipped in. Phosphorylation was expressed as P_i uptake in μ moles per flask.

Method of estimating electrolyte leakage from tissue.--Leakage of electrolytes from toxin-treated and control tissue was estimated from changes in electrical conductivity of glass distilled water in which tissue samples were bathed (59). Treated and control samples were enclosed in cheesecloth bags, rinsed with glass distilled water at 22°C, and shaken at the rate of 90-120 strokes per minute. Measurement of electrical conductivity of the ambient solution was made at intervals with a conductivity bridge (model RC 16 BI Industrial Instruments) and the specific conductivity was expressed as reciprocal ohms (mhos.).

Uptake of amino acids by tissues.--Uptake was defined as the amount of labeled amino acid taken in from the medium and retained within the tissue after 1 hour washing (40). Roots from 4 day old seedlings (100-250 mg) were

treated with toxin (25 µg/ml) and incubated in 1 ml L-leucine-1-C^{14} or DL-valine-U-C^{14} (pH 7.0) at concentrations of 50 mM each for 3 hours with gentle shaking at 22°C. Control tissues were treated the same, except that no toxin was added. Chloroamphenicol (15 µg) was added to the reaction medium to prevent bacterial growth. The roots were then washed under running tap water for 1 hour, blotted gently, placed in 1 ml 95% ethanol in tightly stoppered glass tubes and extracted with shaking overnight. Aliquots (0.1 ml) of the ethanol extract were placed on planchets and the radioactivity was counted with a Nuclear-Chicago gas flow counter.

Incorporation of amino acids.--Cuttings from plants 15-22 days old were allowed to take up toxin solution with the transpirational stream, while control cuttings were allowed to take up water. After suitable exposure times, samples of 100-250 mg were cut from the center sections of 10 leaves (avoiding the mid ribs), placed in 2-2.5 ml labeled amino acid solutions (0.5 µc/ml) and vacuum infiltrated for 15-20 minutes. The tissue samples were then incubated in petri dishes on moist filter paper for 4 hours at 22°C. After incubation, each sample was extracted 3 times with hot ethanol, ground in a mortar with 80% ethanol, centrifuged, and the residue resuspended and recentrifuged 3 times more in 80% ethanol (30). The pellet was then

suspended in 5% TCA at 0°C for 15 minutes, and centrifuged; this process was repeated twice more. The TCA insoluble precipitate was extracted once with 80% ethanol, once with absolute ethanol, and twice with hot ethanol-ether (3:1 v/v). The pellet was suspended in 3 ml 1 N NaOH at 90°C for 1 hour and centrifuged. Amino acid incorporation was determined by radioactivity counts on aliquots. Samples of 0.1 ml were placed on sand-blasted glass planchets, dried, and counted with a Nuclear-Chicago gas flow counter.

Uridine incorporation into RNA fraction.--Cuttings from 15 day old plants were allowed to take up toxin solution (11-13 µg/ml) with the transpirational stream for 8 to 13 hours. Samples (250 mg) were cut from the center sections (1 cm pieces) of 10 leaves from 10 plants, being careful to avoid the mid rib. Leaf sections were vacuum infiltrated in 2.0-2.5 ml of C^{14} uridine solutions (0.5 µc/ml) for 15 to 20 minutes, followed by incubation in petri dishes on moist filter paper for 4 hours. The tissue samples were then extracted 3 times with hot 80% ethanol, ground in a mortar with 10% TCA and left overnight in the cold room in centrifuge tubes. The precipitate was washed 3 times with 5% $HClO_4$ and 2 times with 95% ethanol. After the final centrifugation, pellets were suspended in 2.0 ml of 0.3 N KOH and incubated for 18 hours at 37°C. The solution was

adjusted to pH 1.5-2 with $HClO_4$ to precipitate DNA. DNA and the insoluble $KClO_4$ were removed by centrifugation and the ribonucleotides in the supernatant solution were determined in aliquots of 0.1 ml placed on planchets, dried, and counted for radioactivity as described above.

Cellulase preparation.--Cultures of Myrothecium verrucarie, isolate 460 (obtained from Dr. Mary Mandels of the Quartermaster Laboratory at Natick, Massachusetts) were grown on PDA slants for 8 to 10 days until heavy sporulation occurred. Each slant was then flooded with 10 ml of sterile distilled water, then the spores and mycelium were scraped into suspension with a sterile knife. The suspension from each tube was poured into 100 ml Whitaker's solution (61) containing 0.1 gm glucose and 1.0 gm chemically pure grade Whatman cellulose (39). The cultures were shaken at 120 strokes/minute for 14 days at 22°C. The culture filtrate was then concentrated to one tenth of its volume with a flash evaporator at 37°C. The concentrated filtrate was fractionated at 2°C with $(NH_4)_2SO_4$ (15). The fraction precipitated at 35-70% saturation was redissolved in 1-2 ml glass distilled water and desalted by passing through a 13 x 1.5 cm column of Sephadex G-25 at 2°C. The column was previously washed and developed with 0.1% NaCl in the

cold room. One ml fractions were collected; cellulase activity was found in fraction 2. This enzyme preparation remained active up to four months when kept at -20°C.

Preparation of cell wall free protoplasts.--The method of Ruesink and Thimann (39) was followed. Seedlings were grown in the dark with intermittent dim red light for 72 hours. Epidermal strips were removed from the coleoptiles with jeweller's forceps. Five mm subapical sections were then cut from the remaining tissues and the primary leaf was removed with a thin wire. Sections 1 mm long were then cut and placed in a solution containing 100 µl cellulase (as prepared above) and 100 µl 1.0 M mannitol. After 1 hour, 2 ml 0.5 M mannitol was added. Protoplasts were allowed to settle to the bottom of the tube for 10 minutes and the supernatant solution was removed with a pipet. Mannitol solution was added and the process was repeated, to remove cellulase.

Ribonuclease assay.--Ribonuclease activity was determined spectrophotometrically as described by Tuve and Anfinsen (52). The enzyme was prepared as follows: 1.5 gm leaves were homogenized for 2 minutes in the cold room in a small stainless steel Waring Blendor in 0.1 M potassium phosphate buffer (pH 5.7). The homogenate was stirred for 1.5 hours at 4°C. The insoluble material was centrifuged and discarded. The supernatant solution was allowed to stand

overnight and the precipitate formed was removed by centrifugation. The supernatant fluid was brought to 50 ml (pH 5.1) and aliquots were used for the assay of ribonuclease.

For estimation of ribonuclease activity, 0.5 ml of the enzyme preparation (30 mg fresh wt. tissue/ml) was added to 1 ml yeast RNA (15 mg/ml) in 0.1 M acetate buffer (pH 5.0) and the solution was brought to a total volume of 2 ml with acetate buffer. The reaction mixture was incubated for 30 minutes at 37°C, after which the unhydrolyzed RNA was precipitated with 1 ml 0.75% uranyl acetate in 25% $HClO_4$. The contents were chilled for 15 minutes and then centrifuged. A 0.1 ml aliquot of the supernatant was diluted sixty times. The optical density of the nucleotides released in the reaction was read against blank (for zero time) at 260 mμ using a Beckman DB spectrophotometer. The amount of ribonuclease required to cause an increase in optical density (ΔE_{260}) of 0.01 was defined by Tuve and Anfinsen as one unit of activity (52).

Histological studies.--Sorghum seeds were sterilized in 10% Chlorox for 10-15 minutes, rinsed thoroughly with sterilized distilled water and germinated in sterilized petri dishes on moist filter paper. When the roots reached 0.5 cm long they were inoculated by placing a small piece from a culture of P. circinata on each root tip. Plants were then placed in sterile flasks containing 30 ml White's nutrient solution plus 2% agar. These were incubated at room temperature in diffuse light. At intervals of 1 to 7 days root

tips (0.5 cm) were removed and fixed for 24 hours in formaldehyde (40%), acetic acid, ethyl alcohol fixative (FAA). Root tips were dehydrated in a graded series of ethanol-water, followed by clearing in a graded ethanol-xylene series. Infiltration and embedding were done in paraffin wax following the procedure outlined by Sass (43). Sections 10-12 μ in thickness were cut with a rotary microtome. Sections were stained with fast-green and safranin. The stained sections on slides were examined microscopically for the presence of mycelia in tissues.

RESULTS

Effects of toxin on growth of resistant and susceptible sorghum seedlings.--A highly purified sample of PC-toxin was obtained from Dr. R. B. Pringle of the Plant Research Institute, Ottawa, Canada. The toxin was dissolved in water (2.6 mg/ml) and diluted serially for bioassay, using susceptible (cv. Colby) and resistant (cv. RS-610) sorghum seedlings. Results of the bioassay showed that growth of susceptible sorghum seedling roots was completely inhibited at 0.1 µg/ml, and partially inhibited by concentration as low as 0.01 µg/ml. In contrast, growth of resistant sorghum seedling roots was not inhibited at the highest concentration of toxin used (2.6 mg/ml). Higher concentrations were not used because of the limited supply of pure toxin. Therefore, resistant plants will tolerate more than 26,000 times higher concentration of toxin than is required for complete inhibition of susceptible plants.

PC-toxin is more active on a dry weight basis than HC-toxin, but considerably less active than HV-toxin. HV-toxin gives complete inhibition of seedling root growth of susceptible plants at 0.0002 µg/ml (47), while the

resistant variety tolerated more than 1,000,000 times this concentration with no apparent effect. Recent data with HC-toxin (20) indicated that 0.5 µg/ml is needed to give complete inhibition of seedling root growth of susceptible corn, while inhibition of growth in resistant corn requires 100 times higher concentrations of toxin.

Effects of toxin concentration, exposure time, and temperature on toxicity to sorghum seedlings.--A general idea of the kinetics of toxin uptake may be obtained by studying the effects of toxin concentration, exposure time, and temperature on toxin uptake by treated tissues. The experiments of Scheffer and Pringle on HV-toxin uptake (46) were repeated with PC-toxin. Germinated seeds were left for various times under different conditions in toxin solutions, then were rinsed thoroughly in water and placed in distilled water or fresh nutrient solution. Inhibition of root growth indicated an accumulation of toxin by the treated tissue. Growth of roots should indicate no toxin uptake, or uptake of sub-toxic amounts. Each experiment described below was done 2 or more times.

Toxin concentrations of 1.3, 0.13, and 0.013 µg/ml water were used. Individual groups of germinated susceptible sorghum seeds were incubated in each of the above concentrations for 2.5, 5, 8, 12, or 24 hours. Seeds were taken out of the toxin solutions and washed through 10 changes of water (30 ml each). Each group was then placed

in 5 ml distilled water, in samll petri dishes, and incubated for 4 days as in the standard bioassay. Control susceptible seedlings were incubated in water during the times of toxin treatments, after which they were washed and placed in petri dishes in water. Seeds which were exposed to 1.3 µg toxin/ml acquired a toxic dose in the shortest exposure time, 2.5 hours. With 0.13 µg toxin/ml, the percentage of the seeds that grew roots was 60% after 2.5 hours exposure and decreased to 30% when left in toxin solution for 24 hours (Table 1). This toxin preparation gave complete inhibition of susceptible roots at a concentration of 0.1 µg/ml when the seedlings were left in toxin solution during the whole assay time. Seedlings treated with the 0.013 µg/ml showed no toxic effects and were comparable to control seedlings in water. Eighty percent or more of the seedlings grew roots in control solutions.

Effect of temperature on toxin uptake was measured, using the pyridine eluate from a Norit-Celite column as a source of toxin. This toxin preparation inhibited susceptible root growth at 0.225 µg/ml when seeds remained in the assay solution for the entire assay time. Toxin concentrations of 22.5, 2.25, and 0.45 µg/ml were used. Petri dishes containing toxin solution of the above concentrations were placed in incubators at 5, 21, 32°C and allowed to equilibrate. After equilibration, 150 germinated susceptible sorghum seeds were placed in each toxin

TABLE 1.--Effect of toxin concentration and exposure time on root growth of susceptible (cv. Colby) sorghum seedlings.

Exposure time[a] (hr.)	% of seeds[b] that grew roots after toxin treatment		
	µg/ml 1.3	0.13	0.013
	%	%	%
2.5	20	60	90
5.0	15	50	85
8.0	0	40	80
12.0	0	40	80
24.0	0	30	80

[a]After exposure to toxin solutions for the hours shown, seeds were washed thoroughly and incubated in water.

[b]% of seedlings with roots > 1.0 cm long after 4 days. 80% of non-toxin-treated control seedlings had roots > 1.0 cm long.

concentration at each indicated temperature. After 2.5 hours exposure, seeds were removed, washed, and placed in distilled water at room temperature as described above. Results (Table 2) indicated that at each concentration of toxin the percentage of plants that failed to grow roots was greater at the higher temperature. The expression of toxicity by susceptible tissue increased with increasing toxin concentration and exposure time. Temperature affected toxin uptake since more seedlings failed to grow at each concentration of toxin as the temperature was increased from 5 to 21 to 32°C (Table 2).

Results of the experiments described above are expressed only as the percent of seedlings that grew or failed to grow roots. Such results could be expected if the sorghum cultivar was not genetically uniform, but contained a mixture of very susceptible seeds with seeds of several degrees of tolerance to the toxin. For this reason the average length of the seedling roots that grew more than 2 cm in different concentrations of toxin incubated at the 3 different temperatures (5, 21 and 32°C) was determined. Results show that the average length of the roots that grew did not differ with temperature and toxin concentration (Table 3). These results indicate that the P. circinata susceptible cv. Colby is a genetic mixture containing seeds with several levels of tolerance to the toxin. However, the results of the 3 types of experiments, considered together, show that toxin uptake increases with exposure time, and the rate of uptake is affected by the temperature and toxin concentration.

TABLE 2.--Effect of toxin concentration and temperature during the toxin exposure period on later growth of susceptible sorghum seedlings.

Toxin Conc.[a] (µg/ml)	% of seedlings with roots that did not grow more than 1 cm		
	°C		
	5	21	32
Control (H_2O)	27	26	30
0.45	37	39	53
2.25	35	57	74
22.50	68	80	93

[a]Treatment time was 2.5 hours, and growth was measured after 4 days. The partially purified toxin preparation gave complete inhibition of seedling root growth at 0.225 µg/ml when seedlings remained in toxin solutions. There were 150 seeds in each category.

TABLE 3.--Effect of temperature and toxin concentration on growth of seedlings (cv. Colby) that were not affected by toxin treatment. Many seedlings were affected (see Table 2).

Toxin Conc. (µg/ml)	Average Root Length (cm)[a]		
	Temperature °C		
	5	21	32
	cm	cm	cm
Control (H_2O)	6.4	6.1	6.3
0.225	6.5	7.1	6.6
0.450	6.9	7.04	6.6
2.250	6.7	7.3	8.0
22.500	6.5	5.3	6.08

[a]Treatment time was 2.5 hours. All seedlings were then washed and incubated at room temperature. The partially purified toxin preparation gave complete inhibition of seedlings growth at 0.225 µg/ml, when seedlings remained in toxin solutions. 150 seeds were included in each category. Only the values for roots that grew > 2.0 cm after toxin treatment were included.

Recovery of toxin from treated cuttings.--Toxin recovery from treated susceptible cuttings but not from resistant cuttings led to the hypothesis that the nature of resistance to HV-toxin is based on the ability of intact tissue to inactivate toxin (38). This conclusion has never been confirmed, and Scheffer and Pringle (46) have proposed an alternative hypothesis for resistance, suggesting that resistant tissue lacks a toxin receptor, or toxin-sensitive sites. I have therefore attempted to recover toxin from treated cuttings, with the hope of learning something of the nature of resistance to PC-toxin.

Transpiring cuttings (10 gm each) of 15-22 day old P. circinata resistant and susceptible plants were allowed to take up a solution containing 36.4 µg toxin/ml. Control cuttings were allowed to take up water. After 5 and 21 hours the volume of solution taken up was measured, the cuttings were homogenized in a Waring Blendor, and the total volume was brought to 50 ml with water. Extracts were assayed against susceptible and resistant seedlings, using a dilution series of the homogenized tissue extract. The percent of toxin recovered from ground cuttings was estimated from assay end points. In another experiment the same amount of toxin solution taken up by the transpiring cuttings was added to previously homogenized tissue of both susceptible and resistant plants. This homogenate was then bioassayed against both susceptible and resistant seedlings.

Toxin was detected from both resistant and susceptible cuttings that were allowed to take up toxin solutions before being homogenized (Table 4). The amount of toxin recovered varied from 20 to 72% in 3 different experiments, using both susceptible and resistant cuttings. In each case more toxin was recovered from resistant than from susceptible cuttings. In one experiment, almost twice the amount of toxin was recovered from resistant treated cuttings, after both 5 and 21 hours toxin exposure time (Table 4). Less toxin was recovered after 21 hours than after 5 hours exposure; for example, in one experiment 72% of the toxin taken in by resistant cuttings was recovered after 5 hours, and 48% after 21 hours. Susceptible cuttings had the same trend, with less recovery after the longer exposure time, although the percent recovery was lower for susceptible than for resistant cuttings in all cases. When toxin was added to homogenates rather than to cuttings, there was 100% recovery from homogenates of both susceptible and resistant plants.

These results indicate that susceptible tissues retained or inactivated more toxin than did resistant tissues. The amount retained by susceptible cells was enough to cause injury, which might indicate the presence of certain sites on the surface of susceptible cells that irreversibly adsorbed definite amounts of toxin. On the other hand the smaller percentage retained or inactivated by resistant cells could be attributed to less available sites on their surfaces for toxin adsorption. Regardless of these speculations, it is clear

TABLE 4.--Recovery of PC-toxin from treated cuttings of susceptible (cv. Colby) and resistant (cv. RS-610) sorghum plants.

Cutting Type	Exposure Time[a] (hrs)	Total Uptake[b] (µg)	Total Recovery[c] (µg)	% Recovery
Susceptible	5	72.8	25	34
	21	200.2	52.5	25
Resistant	5	72.8	52.5	72
	21	218.4	105.0	48

[a]Transpiring cuttings took up toxin solution (36.4 µg/ml) for the times shown. The original toxin preparation gave complete root growth inhibition at 0.1 µg/ml.

[b]Uptake/10 grams tissue.

[c]Recovery was estimated by bioassay. Similar results were obtained in 2 other experiments.

that resistance to PC-toxin does not depend on superior ability of the resistant tissue to inactivate toxin, as compared to susceptible tissue. Also, the intact cell is required to inactivate or adsorb toxin, as indicated by 100% recovery of toxin added to plant homogenates.

Effect of toxin on O_2 uptake by tissue.--An increase in oxygen uptake by susceptible sorghum plants treated with PC-toxin has been reported (47), but no data were published. The effects of toxin on respiration of both leaves and roots were determined in a number of experiments. Leaf samples (300 mg fresh weight) were vacuum infiltrated with toxin solution (27 µg/ml) or water for 10 minutes. After infiltration, samples were placed on moist filter paper in Warburg flasks and equilibrated for 10 minutes at 30°C. Oxygen uptake was measured by standard procedures (53). Roots (200 mg) from 4-5 day old seedlings were pretreated with toxin solution (25 µg/ml) or water in Warburg flasks for 5 hours before oxygen uptake was measured. In another experiment roots were pretreated with toxin solution (25 µg/ml) for 4.5 hours, then washed, blotted and placed in Warburg flasks in 2 ml 0.03 M phosphate buffer (pH 5.4).

Results with leaf tissues expressed as oxygen uptake per flask indicated 42% more O_2 uptake by toxin treated than by control leaves (Fig. 2). Comparable results were obtained when data were calculated as oxygen uptake per gram fresh or per mg dry weight. In each case, oxygen uptake after toxin treatment ranged from 42 to 48% above uptake by control

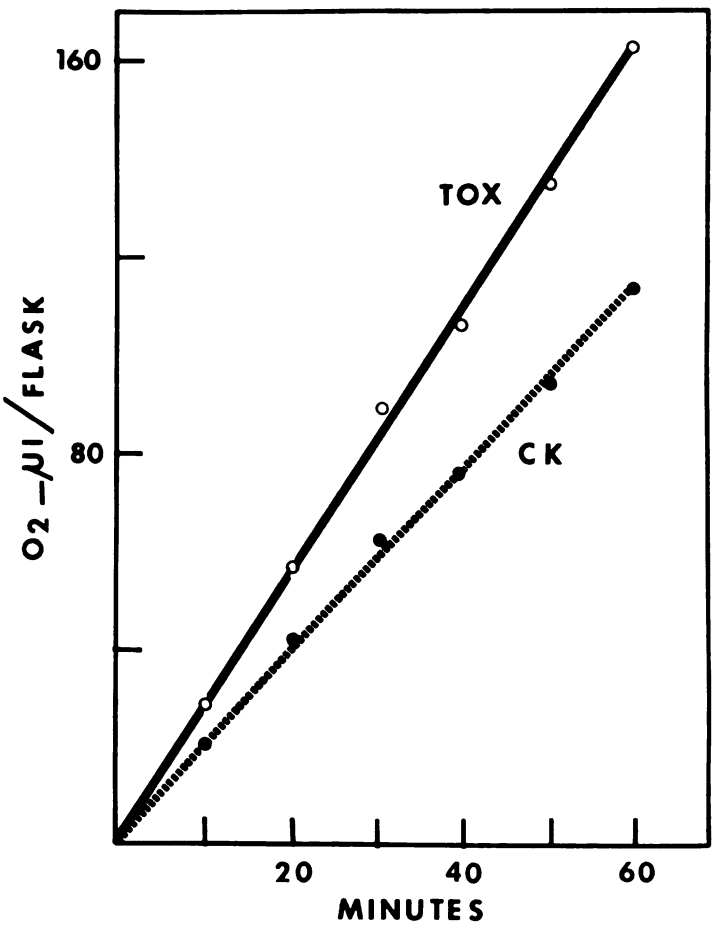

Figure 2.--Effect of toxin on O_2 uptake by Periconia circinata susceptible leaves. Tissue samples (300 mg) were vacuum infiltrated with toxin solution (27 µg/ml) for 10 minutes and placed on moist filter paper in Warburg flasks. Oxygen uptake was determined manometrically at 30°C.

tissue. Toxin treatment of roots resulted in a 10% increase in oxygen uptake over that of the untreated controls, when pre-treated roots were washed before gas exchange was determined. However, when roots were placed in the Warburg flasks without washing and held for 5 hours with no change in the suspending solution, the toxin caused a 20-25% increase in oxygen uptake (Fig. 3). In all cases, roots had a lower respiratory response to toxin than did leaves.

Effect of toxin on mitochondrial oxidation.--The possibility that toxin has a direct effect on the tissue respiratory centers was examined by the use of mitochondria. Oxidative particles were prepared as described in a previous section from susceptible sorghum seedlings. Potassium succinate, 50 μM/flask, was added as a substrate. The cofactors NAD, TPP, CoA and cytochrome c were added for maximum activity (62). Toxin solution was added to the reaction mixture in concentrations up to 6.3 μg/ml. Oxygen uptake was measured manometrically for 50 minutes. The experiment was repeated 3 times.

The results show that PC toxin has no effect on succinoxidase activity by mitochondria (Fig. 4). This indicates that the increase in tissue respiration in response to toxin is not a direct effect on the respiratory centers. Apparently, increase in respiration is a secondary effect of P. circinata infection or its toxin. Increased respiration may be a general response to many disturbances (21, 54).

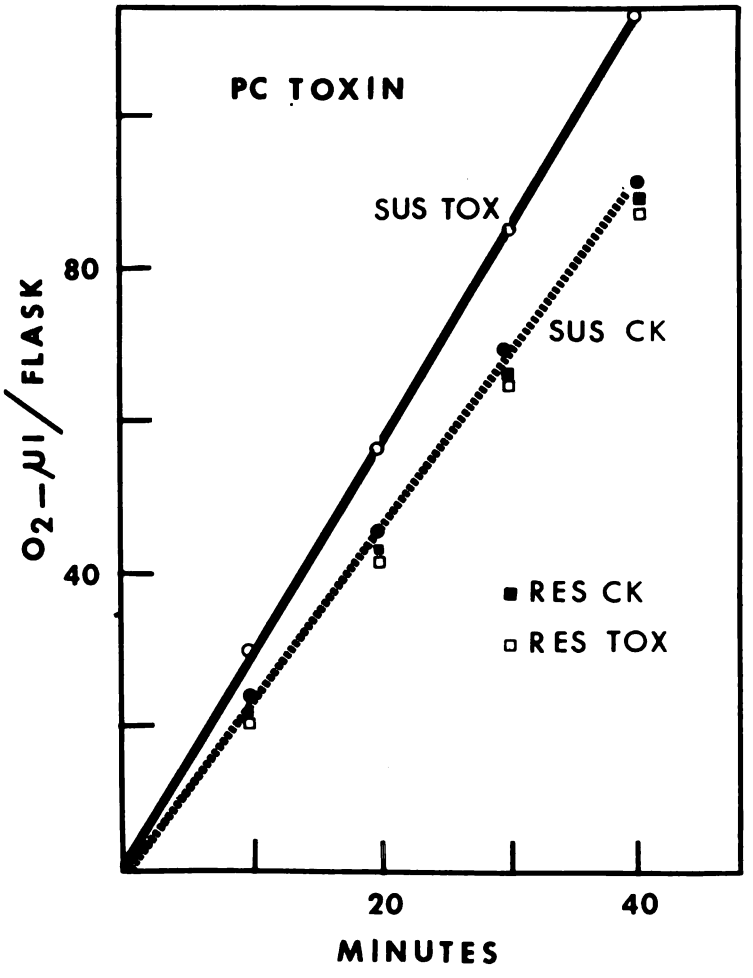

Figure 3.--Effect of toxin on O_2 uptake by resistant and susceptible sorghum roots. Samples (200 mg) were treated with toxin solution (25 µg/ml) in Warburg flasks for 5 hours before measurement. ○ = susceptible treated roots; ● = susceptible control; □ = resistant treated; ■ = resistant control roots.

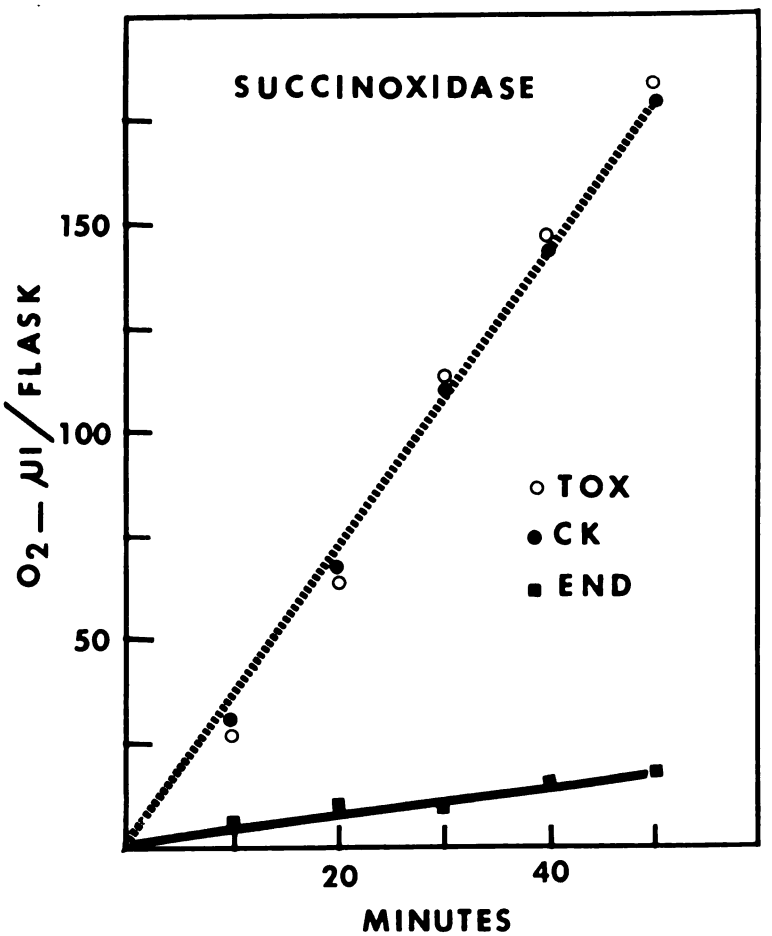

Figure 4.--Effect of toxin on O_2 uptake by mitochondria from P. circinata susceptible sorghum seedlings. Reaction mixture (pH 7.3) contained the following, in µ moles/flask: sucrose, 400; potassium succinate, 50; KH_2PO_4, 140; $MgSO_4$, 15; ATP, 2; NAD, 0.26; cytochrome c, 0.2; CoA, 0.026. Toxin treated (tox), control (ck), endogenous (end) values are indicated.

Effect of toxin on oxidative phosphorylation.--
Uncoupling of oxidation and phosphorylation was once thought to be the cause of respiratory increase in HV-toxin treated tissues (19). This possibility was eliminated by evidence provided by others (45, 60). The fact that mitochondrial succinoxidase activity was insensitive to PC-toxin, as shown in the experiment above, does not rule out the possibility of uncoupling. The significant ablity of mitochondria to incorporate inorganic phosphate into energy-rich phosphate was used to test possible uncoupling effects of PC-toxin.

Phosphorylation efficiency of the oxidative particles prepared from susceptible and resistant sorghum was determined using α-ketoglutarate as a substrate (62). The cyclic reaction beyond succinate was inhibited by adding malonate to the reaction mixture, and hexokinase was added to complete the system. All cofactors used in the previous experiment were also added for maximum activity. Toxin was added to the reaction mixture in Warburg flasks before equilibration. Oxygen uptake was determined manometrically for 40 minutes and phosphate uptake was determined by the method of Fisk and Subbarow (12). Separate duplicate flasks were used to determine starting P_i levels, after equilibration, and before oxygen uptake was measured.

Results from 3 experiments (Table 5) show no significant differences in oxidation or phosphorylation by toxin treated or control mitochondria from susceptible or resistant seedlings. The mitochondria were reasonably well coupled, as shown by P:O ratios above 3.0 in some cases. Theoretically, perfect coupling should give a P:O ratio of 4.0 with α-ketoglutarate as the substrate (57).

Effect of toxin on C^{14} amino acid incorporation into protein.--This effect was determined by measuring the amount of radioactivity retained in the cellular fraction precipitated with TCA. Transpiring cuttings from resistant and susceptible sorghum plants were allowed to take up toxin solutions of either 13 or 27 µg/ml for 4, 8, or 13 hours. Following toxin treatment, randomized duplicate batches of leaf sections weighing 250 mg each were cut from 10 leaves and vacuum infiltrated in 2 ml of valine-C^{14} for 15-20 minutes. After incubation for 4 hours at room temperature, samples were extracted and processed as described in the methods section. Radioactivity in the NaOH hydrolysate was determined by a planchet-counting technique. Incorporation of C^{14}-valine by resistant tissue was not affected by toxin at any concentration or exposure time used. Incorporation by susceptible tissue was affected; tissues pre-treated for 4 hours with toxin at a high concentration (27 µg/ml) caused a 30% decrease in incorporation of C^{14}

TABLE 5.--Effect of toxin on oxidative phosphorylation by mitochondria from sorghum seedlings.

Experiment No	Tissue Type	Treatment[a]	P_i Uptake[b] μ moles/flask	O_2 Uptake[b] μ atoms/flask	P/O
1	Sus.	Control	22.2	8.6	2.7
		Toxin	24.1	7.2	3.3
2	Sus.	Control	26.1	9.9	2.6
		Toxin	28.3	8.5	3.1
3	Sus.	Control	14.3	3.8	3.7
		Toxin	13.1	4.5	2.9
	Res.	Control	15.6	4.7	3.3
		Toxin	15.5	5.8	2.6

[a]Reaction mixture contained the following, in μM/flask: sucrose, 400; α-ketoglutorate, 50; KH_2PO_4, 140; $MgSO_4$, 15; ATP, 2; cytochrome c, 0.2; NAD, 0.26; TPP, 0.26; CoA, 0.026; malonate, 50; and 100 μ moles glucose containing 2 mg/ml Sigma type IV hexokinase. Toxin (final concentration, 3.2 μg/ml) was added before equilibration was started.

[b]P_i values are averages for 4 determinations. O_2 values are averages of 3 flasks each.

valine. A lower concentration (13 µg/ml) decreased C^{14} valine incorporation by 23 and 15% in 8 and 13 hours respectively (Table 6). These results might indicate a direct effect on protein synthesis. The alternative possibility, that toxin might have a direct effect on amino acid uptake and transport and an indirect effect on protein synthesis, was tested in the following experiment.

Effect of toxin on uptake of C^{14}-amino acid.--Amino acid uptake in tissues is a carrier mediated transport across the membrane (17). Active uptake and retention of amino acids by roots treated with toxin were compared with uptake and retention in untreated controls. Roots from 4 day old seedlings were treated with toxin solution (25 µg/ml) for various time periods up to 3 hours, incubated with C^{14}-valine and leucine (0.2-0.5 µc/ml) for 3 hours, washed under running water for 1 hour, and extracted with ethanol. The radioactivity (cpm) in the ethanol extract was a measure of the intracellular free pool amino acids. Results (Tables 7, 8) indicate that toxin caused a decrease in active uptake when tissue was pre-treated with toxin for 1 hour. Pre-exposure to toxin for less than one hour gave no effect on susceptible roots. Susceptible roots exposed to toxin for 3 hours had almost 50% decrease in retention of amino acids within the plasma membranes (Table 8). Uptake and retention of amino acids by resistant roots was not affected by toxin, regardless of exposure time (Tables 7, 8).

TABLE 6.--Effect of PC-toxin on incorporation of C^{14}-valine into TCA-precipitable components of resistant (cv. RS-160) and susceptible (cv. Colby) sorghum leaves.

Exp. No.	Treatment[a]	Exposure Time	Radioactivity[b]		% of Control	
			Sus.	Res.	Sus.	Res.
		hours	CPM	CPM	%	%
1	Control		548	341		
	Toxin	4	386	336	70	98
2	Control		280	160		
	Toxin	8	216	156	77	97
3	Control		222	-		
	Toxin	8	186	-	83	-
4	Control		1058	-		
	Toxin	13	903	-	85	-
5	Control		-	345		
	Toxin	13	-	396	-	111

[a]Fresh weight of tissue samples was 250 mg each. Samples were incubated 4 hours in C^{14}-valine (0.5 µc/ml). Toxin concentration was 27 µg/ml in experiments 1 and 5; 13 µg/ml in experiments 2,3 and 4.

[b]CPM/0.1 ml fractions of NaOH hydrolysate of the TAC-precipitate.

TABLE 7.--Effect of toxin on uptake of C^{14}-valine by susceptible (cv. Colby) and resistant (cv. RS-610) sorghum roots.

Toxin[a] Exposure Time (min)	Treatment[b]	Uptake of C^{14}[c]		% of Control	
		Sus.	Res.	Sus.	Res.
		CPM	CPM	%	%
30	Control	270	234		
	Toxin	273	232	101	99
60	Control	270	234		
	Toxin	231	240	85	102
90	Control	270	234		
	Toxin	140	226	52	96
120	Control	270	234		
	Toxin	144	216	53	92

[a]Tissues were pre-exposed to toxin for the times indicated, followed by 3 hours incubation with valine, in all cases.

[b]Reaction mixture (1.0 ml) contained C^{14}-valine (0.2 µc/ml), 40 m M; 30 m M phosphate buffer (pH 7.0); Chloroamphenicol (15 µg); and 150 mg fresh root tissue. Toxin concentration was 26 µg/ml.

[c]CPM (above background) per 0.1 ml aliquots of the ethanol extract from tissues.

TABLE 8.--Effect of toxin on uptake of leucine-1-C^{14} by susceptible (cv. Colby) and resistant (cv. RS-610) sorghum roots.

Exp. No.	Exposure Time (min)[a]	Treatment[b]	Uptake of C^{14}[c]		% of Control	
			Sus.	Res.	Sus.	Res.
			CPM	CPM	%	%
1	30	Control	203	249		
		Toxin	211	245	103	98
	60	Control	216	232		
		Toxin	181	225	83	97
2	60	Control	2,815	---		
		Toxin	1,913	---	68	--
	120	Control	3,626	---		
		Toxin	2,089	---	57	--
	180	Control	2,805	---		
		Toxin	1,613	---	57	--

[a]Tissues were pre-exposed to toxin for the times indicated, followed by 3 hours incubation with leucine, in all cases.

[b]Reaction mixture (1.0 ml) contained leucine-1-C^{14} (0.2-0.5µc/ml), 50 m M; 30 m M phosphate buffer (pH 7.0); Chloroamphenicol (15 µg); 250 mg fresh root tissue. Toxin concentration was 25 µg/ml.

[c]CPM (above background) per 0.1 ml aliquots of ethanol extract from tissues.

Effect of toxin on incorporation of Uridine-C^{14}.--
The possibility that PC-toxin might affect protein synthesis indicates the need to look for an effect on RNA synthesis, since protein synthesis depends on RNA synthesis. Accordingly, toxin treated and control tissues were incubated with C^{14}-labeled uridine. The effect was determined by measuring the radioactivity retained in the RNA fraction.

Transpiring cuttings were allowed to take up toxin solutions of several concentrations for various exposure times. Samples (250 mg) from 10 different leaves were infiltrated with C^{14}-uridine for 15-20 minutes, incubated for 4 hours and extracted as described in the methods section. Radioactivity in KOH hydrolysate was measured, using aliquots on planchets. Toxin at 13 µg/ml caused a 55% decrease in C^{14}-uridine incorporation by susceptible tissue, as compared with non-treated controls (Table 9), after both 4 and 8 hours pre-exposure times. When the pre-treatment time was 13 hours, toxin at 11 µg/ml caused a 32% decrease in uridine incorporation, as compared with the non-treated control (Table 9). Toxin had no effect on uridine incorporation by resistant leaf tissue. These results could occur from a direct effect on RNA synthesis, or from an effect of toxin on uridine uptake and transport into the cell.

TABLE 9.--Effect of PC-toxin on incorporation of uridine-C^{14} into the RNA fractions from susceptible (cv. Colby), and resistant (cv. RS-610) sorghum leaves.

Exp. No.	Treatment[a]	Exposure Time	Radioactivity[b]		% of Control	
			Sus.	Res.	Sus.	Res.
			CPM	CPM	%	%
1	Control		110	69		
	Toxin	4	50	77	45	111
2	Control		170	-		
	Toxin	8	71	-	47	-
3	Control		98	99		
	Toxin	13	67	109	68	109

[a]Fresh weight of tissue samples was 250 mg each. Samples were incubated 4 hours with Uridine-C^{14}. Toxin concentration was 13 µg/ml in experiments 1 and 2; 11.2 µg/ml in experiment 3.

[b]CPM (above background) per 0.1 ml fractions of KOH hydrolysate.

An apparent decrease in uridine incorporation could result from increased ribonuclease activity in toxin treated tissue. To test this hypothesis, ribonuclease was prepared from toxin treated and non-treated susceptible leaf cuttings by the method of Tuve and Anfinsen (52), as described in the methods section. Assay for RNase in tissue extract was essentially that of Anfinsen et al (5). One ml 0.75% RNA in acetate buffer (pH 5.0), 0.5 ml tissue extract, and acetate buffer to a total volume of 2 ml, were incubated in 12 ml centrifuge tubes. After incubation for 30 minutes at 37°C, the reaction was stopped with 1 ml chilled uranyl acetate solution, and the precipitate formed was removed by centrifugation. To determine hydrolyzed nucleotides in the solution, the optical densities of dilutions of the supernatant were measured at 260 mμ using a Beckmann DB spectophotometer. Each sample was read against its blank which contained the complete reaction mixture, with the reaction stopped at zero time. This was necessary because the enzyme preparation was highly colored. Enzyme activity was calculated as enzyme units per mg fresh weight tissue. As defined by Tuve and Anfinsen (52), "the amount of RNase required to cause an increase in optical density (ΔE_{260}) of 0.01 was defined as one unit of activity."

Toxin had no effect on RNase activity at pH 5.1. Susceptible and resistant toxin treated and control leaves

all had 127 RNase units per mg fresh weight. This level of RNase activity is similar to that found in many plant tissues (8, 52).

Effect of toxin on loss of electrolytes from tissue.--A characteristic feature of infection with many plant pathogens, and of HV-toxin treatment, is an increase in loss of electrolytes from tissues. This effect was studied with PC-toxin, using leaves, roots, and coleoptiles in a number of experiments. Cuttings from plants 15-22 day old were allowed to take up toxin solution (13 µg/ml) for 4.5 hours. Leaf samples (220 mg) were then taken from the centers of 10 leaves from 10 plants for measuring loss of electrolytes to water. Coleoptiles (300 mg) from 10-12 day old seedlings were vacuum infiltrated with toxin solution (13 µg/ml) for 20 minutes, then left for 2.5 hours on moist filter paper in petri dishes before being placed in distilled water to measure electrolyte loss. With roots the treatment varied; in some cases roots from seedlings 4 day old were removed and placed in toxin solution. In other experiments, intact seedlings were treated, then the roots were removed before electrolyte loss was measured. In every case, the samples were enclosed in cheesecloth bags after toxin treatment, rinsed in glass distilled water, and placed in flasks containing 100 ml glass distilled water on a shaker. Conductivity of the water was measured at intervals.

Coleoptile tissues released electrolytes more readily than did leaf or root tissue. After 3 hours leaching, the water containing treated coleoptiles had more than twice the conductivity of the water which contained control tissue (Fig. 5). The specific conductance of the ambient solution containing treated susceptible leaf tissues was about double the conductivity of the solution containing control leaf tissue after 4.5 hours of leaching (Fig. 6). Loss of electrolytes was much less from root tissue than from leaves, but the solutions containing treated roots had 20% higher conductance than ambient solutions of controls after 3 hours. Leaves, roots, and coleoptiles of the resistant plants were not affected by toxin as measured by electrolyte loss (Fig. 6).

H. victoriae toxin causes a very rapid loss of electrolytes from treated susceptible oat tissues, with a significant effect 5 minutes after toxin treatment (60). Specific conductance in the ambient solution of treated susceptible tissue was 5 times greater than conductance of any other treatment solution in 8 hours. All soluble electrolytes in susceptible leaf tissue were lost 8-12 hours after toxin treatment, suggesting a destruction of the plasma membrane and the tonoplast (41). With H. carbonum toxin the rate of loss of electrolyte was slower than with HV- and PC-toxins. Leaves pre-treated with HC-toxin for 4 hours did not show a difference from non-treated

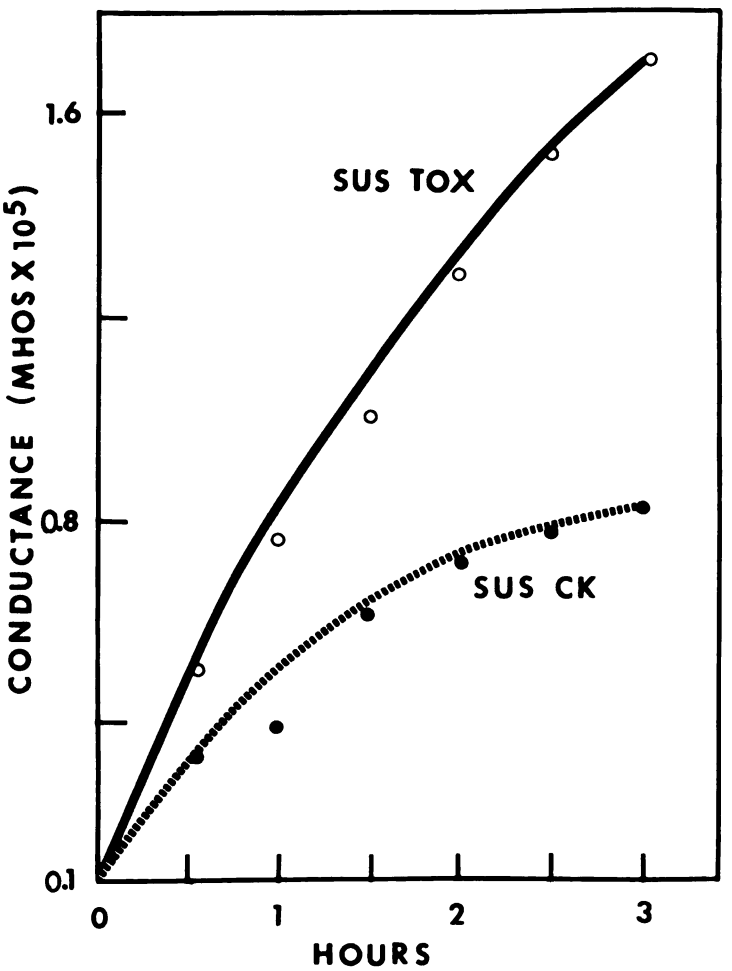

Figure 5.--Effect of toxin on loss of electrolytes from P. circinata susceptible coleoptiles. Tissue samples (300 mg) were vacuum infiltrated with toxin solution (13 µg/ml) for 20 minutes, left on moist filter paper for 2.5 hours, then suspended in glass distilled water. Electrolyte loss was determined from conductivity of the bathing water.

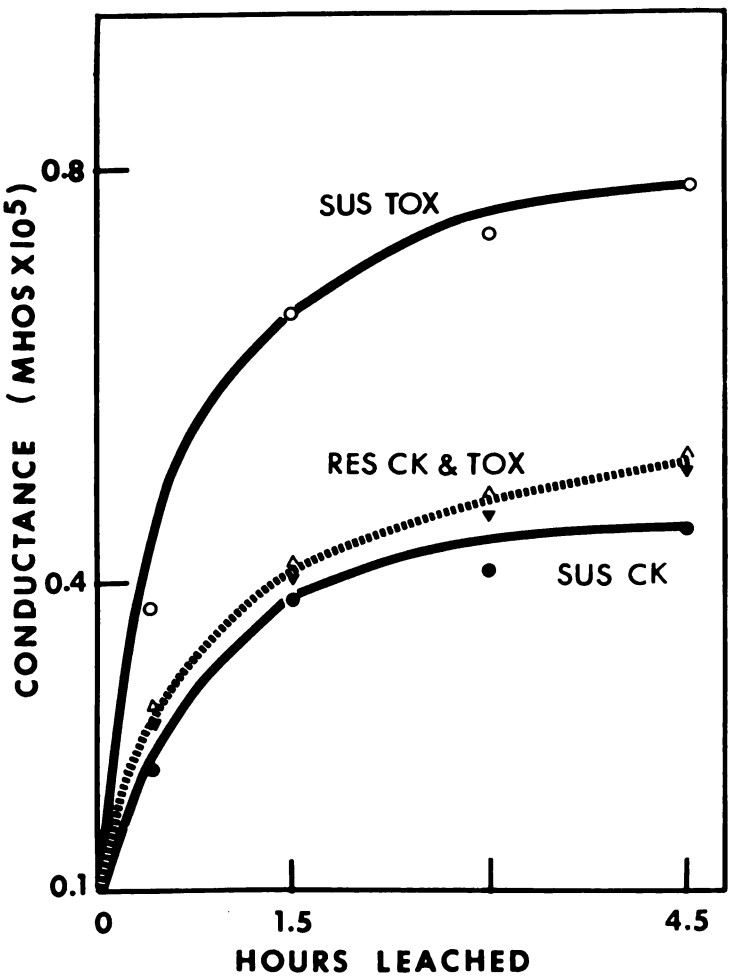

Figure 6.--Effect of toxin on loss of electrolytes from susceptible and resistant sorghum leaves. Transpiring cuttings took up toxin solution (13 µg/ml) for 4.5 hours. Samples (220 mg) were cut from the leaves and suspended in glass distilled water. Electrolyte loss was determined from conductivity of the bathing water. O = susceptible treated; ● = susceptible control; Δ = resistant treated; ▲ = resistant control.

controls when the conductivity of the ambient solutions was measured. However, an 8 hour treatment of susceptible corn leaves with HC-toxin gave a significant increase as compared with non-treated controls (20).

Effect of toxin on cell wall free protoplasts.--An attempt was made to study the effect of toxin on cell wall free protoplasts, following previous work with the HV-toxin model. The effect of HV-toxin on naked protoplasts was dramatic. All susceptible oat protoplasts were destroyed within 1 hour by HV-toxin treatment (41, 42).

Protoplasts from PC-toxin resistant and susceptible sorghum coleoptiles were prepared by the method of Reusink and Thimann (40). Protoplast suspensions (10 µl each) were placed on depression slides, and various concentrations of PC-toxin or DNP were added in equal volumes. The basic reaction mixture consisted of 10 µl protoplast suspension plus 10 µl buffered test solutions. Spherical protoplasts were counted at zero time under the microscope, and slides were held in moist chambers for survival counts at the needed time intervals. Survival was calculated as percentage of the zero time count.

Protoplasmic streaming was very hard to see in the cell wall free protoplasts. A few showed cyclosis, but most were so filled with large granules of refractive plastids that streaming was impossible to observe. A high

concentration of toxin caused only a few of the protoplasts from susceptible sorghum to burst. A decrease of only 14% in protoplast survival was detected after 3 hours toxin treatment. Toxin-treated resistant protoplasts had as good survival as did the controls, with about 5% loss in 3 hours (Table 10). Observation for more than 3 hours was not practical because of the fragility of protoplasts; the spherical form of both control and treated protoplasts was lost. No effects of toxin on protoplasmic streaming were observed. Several protoplast preparations were examined with the same results, using 3 different cellulase preparations. In contrast to the effect of PC-toxin on free protoplasts of sorghum, HV-toxin caused 100% bursting of susceptible oat protoplasts in only 1 hour (42). It also stopped cyclosis or protoplasmic streaming within 10 minutes after exposure to toxin.

Toxin treated and DNP (2×10^{-4} M) treated protoplasts were compared. Results (Table 11) indicate that DNP affected susceptible and resistant protoplasts almost equally, whereas toxin caused some destruction of susceptible protoplasts but no destruction of resistant sorghum protoplasts. DNP acted a little faster than did the toxin. After one hour exposure there was a greater loss of DNP-treated susceptible protoplasts than toxin-treated protoplasts. However, DNP affected both susceptible and resistant protoplasts equally.

TABLE 10.--Effect of PC-toxin on survival of protoplasts from resistant (cv. RS-610) and susceptible (cv. Colby) sorghum coleoptiles.

Protoplast Type	Treatment[a]	Protoplast Survival After[b] Minutes			
		30	60	120	180
		%	%	%	%
Susceptible	Control	100	95	96	95
	Toxin	93	86	81	82
Resistant	Control	100	95	95	93
	Toxin	94	92	92	89

[a]Protoplast suspending medium contained 0.5 M mannitol buffered at pH 6.5 with phosphate. Buffered treatment solutions (10 µl each) were added to 10 µl of protoplast suspensions. Toxin solutions were 0.64 mg/ml.

[b]Calculated as percentage of intact protoplasts at zero time. From 30 to 70 protoplasts were observed for each determination.

TABLE 11.--Comparative effects of PC-toxin and 2, 4-Dinitrophenol on protoplasts from resistant (cv. RS-610) and susceptible (cv. Colby) coleoptiles.

Protoplast Type	Survival After One Hour[a]		
	Control	Toxin	DNP[b]
	%	%	%
Susceptible	95	86	73
Resistant	95	92	76

[a]Calculated as percentage of intact protoplast at zero time. From 30-70 protoplasts were observed for each determination.

[b]Protoplast suspending medium contained 0.5 M mannitol buffered at pH 6.5 with phosphate. Equal volumes of buffered toxin solutions (0.64 mg/ml) or 2, 4-dinitrophenol (2×10^{-4} M) were added to the protoplast suspension. Controls contained buffer only.

PC-toxin appears to act much more slowly than does HV-toxin. Therefore, free protoplasts may not be suitable for studying the action of PC-toxin, since sorghum protoplasts are so fragile that they cannot be kept for more than 3 hours at room temperature. Other kinds of data indicate that more than 3 hours is needed for PC-toxin to act.

The effect of toxin on protoplasmic streaming was observed in root hair cells. Roots of 4 day-old seedlings were treated in small petri dishes with toxin solution (0.64 µg/ml) plus sucrose, while controls were treated with an isotonic solution of 0.25 M sucrose. Treated roots were transferred to slides in a drop of 0.25 M sucrose solution for examination each hour under the microscope. In contrast to the effects of HV-toxin on oats, PC-toxin did not affect protoplasmic streaming in susceptible sorghum root hairs, even after exposure for 6 hours.

Possible effects of gibberellic acid on action of toxin.--The possibility of a toxin-gibberellic acid interaction during seedling growth was tested. Gibberellic acid was used at several concentrations either before or after germinated seedlings were treated with toxin. One group of seeds was allowed to germinate for 24 hours, then was treated with toxin (1.3 µg/ml) for 2.5 hours. They were then washed through 10 changes of water (20 ml each).

divided into sub-groups and incubated in solutions containing gibberellic acid at 10^{-4}, 10^{-5}, 10^{-6}, or 10^{-7} M for 2.5 hours. Seedlings were washed again as described above and grown for 4 days in water. Another group of seedlings was first treated in GA, washed, treated with toxin, washed again, and finally incubated in water for 4 days. Resistant seedlings treated as described above were used as controls. Another control was kept in water, while still others were incubated in the several concentrations of GA.

GA did not counteract or enhance the effects of toxin, whether used before or after toxin treatment. All toxin treated seedlings had inhibited root growth, whether GA was used or not.

Histology of infection. While these studies were underway, statements appeared in the literature to the effect that P. circinata is not a typical tissue invader, but is a soil borne saprophyte which kills its host by the action of its exotoxin (13, 56). No data were given or cited in support of these statements. Therefore I have attempted to answer the question of whether or not P. circinata is a typical tissue invader by the use of standard histological procedures, as described previously.

Microscopic examination of the stained sections showed the presence of mycelial fragments in the cortex.

Twenty-four hours after inoculation the mycelia were seen in the second cell below the root surface. After 48 hours they were found deep within the cortex, almost to the pericycle. By the fourth day after inoculation, the fungus had increased and had reached the pericycle. I did not detect the fungus inside the stele in any case up to 7 days after inoculation. No host tissue disintegration or disruption was detected before the fourth day after inoculation. P. circinata invades tissue in a way typical of many pathogens, but it appears to be a slow grower in its host.

CHAPTER V

DISCUSSION

The host specific toxins of *P. circinata*, *H. victoriae* and *H. carbonum* are being used as models for the study of disease development and disease resistance in plants (47). These substances will reproduce the symptoms of infection by the three fungi, and are required for pathogenicity (34). Loss of ability to produce toxin is always associated with loss of pathogenicity of the organism (47). These facts suggest that the toxins are involved in the initial host-pathogen interactions. Histological studies with *H. victoriae* (31) and *H. carbonum* (18) indicated a typical host-pathogen interaction. Nevertheless, Yoder (63) obtained evidence, with a combination of histological and physiological techniques, that *H. victoriae* toxin is involved in the initial effects on the host. Results of my histological examination of *P. circinata* infection indicate that the fungus invades the tissue without host tissue disintegration for the first four days following inoculation. It is evident that *P. circinata* is a typical tissue-invading fungus, as are *H. victoriae* and *H. carbonum*.

A germinating spore can release only very small quantities of any substance. Therefore, any compound

required for pathogenicity and invasion of susceptible plant tissue must be very active. HV-toxin at 0.0002 µg/ml is known to give complete inhibition of growth of susceptible oat seedling roots. Resistant roots will tolerate more than 1,000,000 times this concentration (47). My data confirm previous reports (47) that PC-toxin gives complete inhibition of susceptible sorghum seedling roots at 0.1 µg/ml, and partial inhibition at 0.01 µg/ml. The tolerance level for resistant sorghum was not completely established because of insufficient amount of highly purified toxin, but it is more than 26,000 times higher than for susceptible plants. Both HV- and PC-toxins are thus very active by any biological standards. HC-toxin is considerably lower in relative activity, and there is less contrast in sensitivity of resistant and susceptible plants. It completely inhibits susceptible corn seedling roots at 0.5 µg/ml, and gives comparable inhibition of resistant corn roots at 50 µg/ml (20). The evidence for HC-toxin as a significant determinant of disease is nevertheless convincing (20, 47, 49).

The amount of PC-toxin taken up by susceptible seedlings was found to be affected by toxin concentration and exposure time. Unlike the case with HV-toxin, where uptake appears to be a simple process (metabolically and temperature independent) (46), the rate of PC-toxin uptake by susceptible tissue is increased by increasing the

temperature. This was shown by brief exposures of seedlings to toxin at either low or high temperatures; later growth by the seedlings exposed to toxin at the high temperatures was inhibited more than was growth of seedlings exposed to toxin at a lower temperature. I have no data on the effect of anaerobic conditions and metabolic inhibitors on PC-toxin uptake; such data are needed for a better understanding of the process of uptake. In contrast to HV-toxin uptake, HC-toxin uptake is metabolically dependent (20).

Susceptibility and resistance to the diseases caused by the microorganisms which produce the host-specific toxins is based on reaction or lack of reaction with the corresponding pathogen-produced determinants (47). In other words, resistance to disease is the same as resistance to the host-specific toxin. There are extensive data, both genetic (49) and physiological (48), showing that specific pathogenicity by H. victoriae isolates is determined by the ability to produce toxin. When H. victoriae isolates lost specific toxin producing ability, pathogenicity was lost also in all cases. Therefore the nature of resistance to toxin becomes the important question, and it is not yet clarified.

Romanko (38) has suggested that resistance to HV-toxin is based on the more efficient ability of intact resistant oat tissue to inactivate toxin, as compared to

susceptible tissue. This suggestion was based on experiments using cuttings that took up measured amounts of toxin solutions. However, the toxin detected in treated susceptible cuttings was less than 8.0% of the toxin taken in. On the other hand, 90-99% of the toxin activity in original solutions was said to be lost, whether susceptible or resistant plants were used (38). These results could not be confirmed (46), and an alternative hypothesis was suggested. Scheffer and Pringle (46) have suggested that resistance to HV-toxin depends on lack of certain receptor or toxin-sensitive sites which are present in susceptible oat tissue. Data from my study clearly demonstrate that resistance to PC-toxin does not depend on the superior ability of the resistant tissue to inactivate toxin. I was able to recover 50 to 70% of the toxin taken up by resistant sorghum cuttings. In each of a number of experiments, less toxin was recovered from susceptible cuttings than from resistant cuttings. I suspect that resistant tissue may have fewer sites affected by toxin than are present in susceptible tissue. When tissue is homogenized, these sites appear to be destroyed, as indicated by the fact that all toxin was recovered when it was added to homogenates of both resistant and susceptible tissue. If the receptor is in the plasma membrane, destruction of this structure might be expected to destroy toxin-binding capacity.

A typical and general response to toxin treated plants as well as to many plant infections is the increase in respiratory rates. Several possible explanations have been given for this increase, including increased activity of normal metabolic pathways, shifts to new pathways, activation of specific enzymes, and uncoupling of oxidation from phosphorylation (54). My data show a 42 to 48% increase in respiration after PC-toxin treatment of susceptible leaves, but no effect on respiration of resistant leaves. However, isolated mitochondria had no response to toxin treatment. Succinoxidase activity, α-ketoglutarate dehydrogenase activity, and phosphorous uptake by isolated mitochondria were not affected by toxin treatment. Since PC-toxin has no effect on the activity of isolated mitochondria, the increase in respiration of the toxin treated tissues cannot be attributed to a direct effect of toxin on respiratory centers. Apparently some system or systems other than the Krebs cycle is the primary site affected by toxin. This in turn could lead to increased respiration.

Another response to PC-toxin in susceptible toxin-treated tissues is decreased incorporation of C^{14}-amino acids into the TCA-insoluble cellular fraction. This could be based on a breakdown in transport of amino acids to the sites of protein synthesis, and such an effect was demonstrated. However, a direct effect on protein synthesis

cannot be ruled out without data on the effect of toxin on cell-free ribosomal preparations from sorghum tissues. Similarly, PC-toxin caused a decrease in incorporation of C^{14}-uridine into RNA. My data rule out the possibility of explaining this as a stimulation of RNase activity in toxin treated tissues. The activities of RNase prepared from toxin treated and non-treated control leaves were identical. Again, the decrease in incorporation of uridine into RNA could result from a breakdown in the transport system, resulting in decreased uridine at the site of RNA synthesis.

A marked reduction in active uptake of amino acids was observed when susceptible tissue was treated with PC-toxin. It is possible that PC-toxin might affect sites on the cell surface which are responsible for active transport across the membrane, therefore affecting the permeability characteristics of the cell to amino acids and uridine. This in turn could affect incorporation of labeled amino acids and uridine into TCA-insoluble substances. The effect of PC-toxin on active uptake of amino acids appears to develop more slowly (Table 7) than does the comparable effect of HV-toxin (40).

Loss of intracellular materials has been reported for H. victoriae and many other infections. A similar response results from HV-toxin treatment, suggesting a drastic change in the cell membranes (51, 59). This

effect could be an important factor in initial establishment of the pathogen in host tissue, and in disease development. Furthermore, Thatcher has suggested that decreased permeablity discourages development of the pathogen (51). Loss of permeability barriers might alter protoplasmic compartmentalization, which could lead to disturbances between cellular enzymes and substrates. Such disturbances could lead to increased respiration and other metabolic changes associated with infection. Increased respiration has been reported as a result of membrane damage (29).

Increased loss of electrolytes from PC-toxin treated susceptible but not from treated resistant tissue was determined by measuring conductivity of the solution in which the treated tissues were bathed. Loss of electrolytes is a very quick response of susceptible oat tissue to HV-toxin (60). The loss of electrolytes from susceptible tissue appears to be more rapid with HV-toxin than with PC-toxin. HC-toxin causes a similar response in susceptible tissue, but it is considerably delayed (20). Amador and Wheeler (4) suggested that increased respiration could result from loss of certain ions, associated with changes in cell permeability. However, there were no conclusive data.

There is good evidence that the primary lesion of HV-toxin is in the plasma membrane (42). Membrane damage

by HV-toxin was demonstrated by its effect on cell wall free protoplasts from susceptible oat coleoptiles, where 100% bursting occurred in only one hour (41). Data of my study show that this system is not adequate for comparable conclusions with PC-toxin. The chief difficulty is that sorghum protoplasts are so fragile that they disintegrate before the slow-acting PC-toxin has its effect. The maximum time limit for reliable data on sorghum proroplasts is about 3 hours, and in this time PC-toxin causes only about 14% of the treated susceptible protoplasts to break. This is somewhat greater than the breakage that occurs in protoplasts from resistant sorghum. There are several other indications in this study that PC-toxin is much slower and less dramatic in its effects than is HV-toxin. Nevertheless, the indications are that PC-toxin has a significant effect on the plasma membrane. It is possible that this structure is changed in subtle ways which affect permeablity without leading to the drastic disorganization expressed by bursting.

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