THE EFFECT OF PLANT EXUDATES ON COTTON SEEDLING DISEASE INCITED BY *PYTHIUM* SP.

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PYTHIUM SP.

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CHAPTER I

INTRODUCTION

Cotton (Gossypium hirsutum L.) is an important agricultural crop in Oklahoma. Seedling diseases are responsible for at least an estimated 3% reduction in yield each year in this state (25). Fungi involved in the cotton seedling disease complex include Rhizoctonia solani Kuehn, Pythium sp., Fusarium sp., and Glomerella gossypii Edg. (3, 34, 44, 54, 58).

Ray and McLaughlin (41) reported that R. solani was the most important pathogen in the seedling disease complex in Oklahoma. Arndt (3) first reported on Pythium ultimum Trow as a pathogen of cotton seedlings and Brinkerhoff, et al. (8) made the first isolations of Pythium sp. from cotton seedlings in Oklahoma.

Seedling diseases of cotton are generally more severe during cool, wet weather than during warm, dry weather (4, 25). It has been demonstrated that at low temperatures the amount of exudate released from roots is greater than at high temperatures (19, 24, 54, 59). These exudates from underground plant parts include amino acids, carbohydrates, vitamins, enzymes, nucleotides, organic acids and inorganic ions (49). The composition of exudates reportedly varies with conditions of the test and during the life of plant; also exudates produced under the same conditions and from plants of the same age may differ in composition between plant species and varieties.

Guinn and Hunter (21) suggested that cotton seedling diseases are generally more severe during cool than during warm weather, because (a) low temperatures slow growth of the cotton seedling more than they slow growth of the disease organisms, (b) the seedlings remain in a susceptible stage of development for a longer period of time, and (c) biochemical changes in the cotton seedling make it a more suitable substrate for the disease organisms. They found that when plants were chilled at 15°C the sugar content of roots increased and suggested this change could increase disease indidence by providing more food for pathogenic microorganisms (20). Vancura (57) found that exudation of some sugars from maize and cucumber was greater at 19°C than at 28°C, and the total exudation was several times greater from roots at low temperatures than at higher temperatures. He concluded that the permeability of the cells, or its regulation, or both were disturbed.

A study by Husain and McKeen (26) on that of Rhizoctonia fragariae Husain ¢ McKeen which attacks strawberry roots at low, but seldom at warm soil temperatures indicated that the exudate from roots of plants grown at 5° or 10°C stimulated both spore germination and mycelial growth of the pathogen. Exudate from strawberry plants grown at 20° and 30°C did not have this effect. They concluded that a considerable quantity of amino acids were present in the low temperature exudates, but not in the high temperature exudates. Schroth, et al. (51) also suggested that at low temperatures the exudation of amino acids increased from germinating seeds of bean, pea, and cotton. They considered that any factor which affects exudation could influence the incidence of disease.

Nezgovorov (37) found that the effect of low temperature on increased incidence of the seedling disease caused by Pythium sp. was partly related to an unknown substance produced by the host plant which stimulated growth of Pythium in Czapek's medium at 10°C, but not at 26°C. He also suggested that low temperatures also increased production of toxins by the fungus and decreased the activity of antagonistic organisms in soil. Schulz and Bateman (52) mentioned that the seeds of pea, bean, cucumber, and corn were rendered more susceptible to attack by R. solani by an initial germination temperature of 5°C for the first 24 hr prior to transfer to normal germination temperatures (22-30°C). Hayman (22) suggested that the increased susceptibility of cotton seed to R. solani when the seed were germinated at a suboptimal temperature was attributed to an increased exudation of organic constituents by seeds at the low temperature. Kidd and West (28) showed that when seeds were soaked in free water at a suboptimal germination temperature for up to 24 or more hours they lost more exudates than similar seeds did when soaked at a higher temperature. Rajagopalan and Bhuvaneswari (39) showed that seeds release a considerable amount of amino acids and sugar during the first 72 hr of germination.

Plant exudates affect plant pathogens directly by inducing spore germination and by contributing to their nutrition. The germination and growth of chlamydospores of the bean root rot fungus, Fusarium solani (Mart.) App. & Ws. f. phaseoli (Burk.) Snyd. & Hans., were favorably affected by host exudates (amino acids and sugars) (50). Rovira (45) showed that the inclusion of exudates from the roots of peas and oats in liquid media increased the growth of microorganisms.

This stimulation was greater for microorganisms from the rhizosphere than for those from outside this zone. Buxton (10, 11) found that the rhizosphere soil extract from susceptible peas increased the spore germination of Fusarium oxysporum Fr. f. pisi (Linf.) Snyd. & Hans.; on the other hand the rhizosphere soil extract from resistant peas decreased the germination of spores of this fungus. Buxton (13) also found that in dilute sucrose or soil-extract media, root exudates of banana from a susceptible variety promoted spore germination of the Panama wilt fungus, Fusarium sp.; exudates from a resistant variety did not promote germination in these two media. He demonstrated that root exudates of resistant plants and susceptible plants contained different amino acids. In exudates of susceptible plants no detectable cysteine or threonine was found; in exudates of resistant plants no leucine, serine or tyrosine was found. Root exudates from young resistant plants contained more aspartic acid, glutamine and proline than those from young susceptible plants, but this difference decreased as the plants aged. Sulochama (55) also suggested a difference of composition of root exudates from resistant and susceptible cotton varieties. Flentje (17, 18) demonstrated that exudation of sucrose was a major factor governing susceptibility of pea to pre-emergence damping-off caused by Pythium debaryanum Hesse. Peas which were susceptible to damping off released greater amounts of sucrose during seed germination than did less susceptible varieties. He mentioned that when planting susceptible seeds with resistant ones, or by adding the exudates from susceptible seeds to soil in which resistant seeds were planted, he found that resistance broke down and that the seeds of the previously resistant variety were rapidly invaded.

Schroth and Cook (48) reported that the greatest seed exudation of bean occurred with the variety most susceptible to pre-emergence damping-off (caused by R. solani and Pythium sp.). Exudation of ninhydrin-positive, silver nitrate-positive, and ultraviolet fluorescing substances was greatest with the variety most susceptible to damping-off, whereas the least exudation occurred with the most resistant variety. These exudates they felt influenced the incidence of pre-emergence damping-off by providing fungi with nutritive substances necessary for germination and growth in soil.

Yung (62) suggested that pea root exudate increased the percentage of germination and germ tube growth of zoospore cysts of Pythium aphanidermatum (Edson) Fitzp. He also found that germination and growth were promoted when pure components of exudates were used in place of the root exudates. Glucose in the presence of one of the organic acids was more effective in promoting germination and growth than either one alone. A similar result was also found by Agnihotri and Vaartaja (2). In pine seed exudates, 14 amino acids, 4 sugars, and 3 organic acids were identified. They found that a mixture of sugars and a mixture of 3 or 4 amino acids effectively stimulated growth of mycelium and germ tubes.

In some cases, fungi need specific nutrients to support their infection (2, 11) or to increase their virulence (31, 59). Weinhold and Bowman (59) suggested that the amount of nitrogen affected the virulence of R. solani. Kraft and Erwin (31) found that when zoospores of P. aphanidermatum were supplemented with exudate, virulence of this fungus to mung bean seedlings was increased on water agar.

Actually, in natural conditions, root exudates may have a negative

effect on the pathogen by increasing the activity of antagonistic organisms in soil (9, 12). Burke (9) has reported that suppression of Fusarium root rot of beans in certain soils was microbiological in nature. Buxton (12) also indicated the importance of antagonists in suppressing growth of Fusarium.

On the other hand, plants may contain or release in exudates substances that are toxic to pathogens. One of the first investigations of this nature was by Reynolds (43) who reported that extracts of flax plants resistant to wilt depressed growth of Fusarium. He concluded that at least part of the resistance could be attributed to a glucoside. Timonin (56) found that several species of pathogenic fungi, including F. oxysporum Schlecht. f. lini (Bolley) Snyd. & Hans. were depressed more in the rhizosphere of a wilt resistant variety of flax than in a wilt susceptible variety. He suggested that this difference was due to the exudation of greater amounts of HCN in the rhizosphere of resistant plants. Rangaswami and Balasubramanian (40) also found that the bacterial population was reduced in the rhizosphere of some varieties of sorghum that had a high HCN content in the roots.

Temperature appears to exercise an important influence on the amount of exudation and also on disease development. Exudates from plant roots in soil supply nutrients to pathogenic organisms resulting in increased growth, or produce toxic materials to suppress the activity of the pathogen.

However, increased growth or virulence of plant pathogens resulting from increased root exudates has not as yet been conclusively demonstrated to actually be the cause of higher disease incidence under conditions which result in increased root exudation.

The purpose of this research was to study (a) the quantitative differences of total sugar concentration, amino acid concentration, and nitrate ion concentration in plant exudates and extracts, (b) the qualitative differences of sugar content in exudates and extracts, (c) growth of Pythium in low and high temperature exudates, and (d) the effects of adding exudate on seedling disease development in infested soil.

CHAPTER II

ISOLATION AND PATHOGENICITY TESTS

Materials and Methods

(1) Isolation:

The Pythium isolates were obtained from soil from a field where cotton had been grown.

Three media were used in isolating the fungus: water agar, rose bengal agar (RBA) (27), and gallic acid medium (19). The cotton-field soil was brought indoors, mixed with cotton seeds and put into pots (40 seeds/pot) which were kept in a growth chamber at a constant temperature of 20°C. The pots were watered every day. After 2-3 days, the seeds were separated from the soil. Seeds which showed signs of rotting were surface-sterilized by immersing in 10% clorox, and then were placed on petri dishes of the above media.

A trapping technique with eggplant (Solanum melongena L.) was also used for isolating Pythium. Fresh and unwounded eggplant fruits were chosen for this purpose. The eggplants were surface-sterilized with 70% ethanol, then slits were cut about 1 cm long and 1 cm deep on a 30° angle using a sterile knife. The infected material (seed pieces or soil) was inserted into these slits using sterile forceps. (Both sterilized and non-sterilized seed was used). In order to avoid contamination from one isolate to another, it was necessary to keep a distance of about 5 cm between the holes on the

eggplant fruits. The eggplants were incubated in a moist chamber at 30°C. Pythium grew faster than other microorganisms in the eggplant. A water-soaked area covered with white mycelium surrounded the inoculation site 3-4 days after the eggplant was placed in the incubator. The inoculated area was surface-sterilized with 70% ethanol, the epidermis of the eggplant removed and a piece of the diseased tissue transferred to a RBA or potato dextrose agar (PDA) plate. Usually oospores of Pythium could be seen in the eggplant tissue by placing a tiny piece of tissue on a glass slide and examining with a microscope.

(2) Pathogenicity test of isolates:

The purpose of this test was to select isolates with several levels of virulence for later experiments. The inoculum was grown on PDA plates at 25°C. After 4 day's incubation, each agar plate was blended with 200 ml of sterile water for 30 seconds. A breeding strain, #15, (PeeDee x BR Westburn) of cotton from the Oklahoma Agricultural Experiment Station was used in this experiment.

Enough pots of soil were prepared and sterilized so that there were six replicates for each treatment or a total of 36 pots. Before inoculation, the soil of partially filled pots was moistened and then 50 ml of inoculum was added evenly over the soil surface. The inoculum was then covered with 0.2-0.3 cm of soil and the seeds placed on top. Finally, the seeds were covered with 2-3 cm of soil and the inoculated pots placed in a growth chamber at a temperature regime of 30°C days (30 D) and 20°C nights (20 N). Days were 14 hr long and nights 10 hr. The seed germination was calculated 2 wk after inoculation, and the root examined for infection after the final count.

Results

The isolates were easily contaminated by other fungi or bacteria when the media of water agar, RBA or gallic acid agar were used to isolate Pythium. It was found that the trapping technique with eggplant fruits was the best method to isolate pure cultures of Pythium from soil or cotton seeds. Five isolates from eggplant, P6, P7, P9, P10, and P11) were tested for pathogenicity.

Pythium rotted the seed or attacked the radicle of cotton seed after the cotton seed germinated. If the damage to the seed was not serious or the development of disease was slow, the seedling survived and continued to grow. However, compared to the non-inoculated seedlings (check), all infected seedlings were stunted.

All five isolates were pathogenic to cotton seed of the Oklahoma strain #15 (Table I). When inoculated with P9 and P10 no seedlings emerged; P6 and P7 were moderately virulent isolates; P11 was the least virulent isolate.

TABLE I

PERCENT OF SEEDLINGS SURVIVING IN SOIL INFESTED WITH FIVE ISOLATES OF PYTHIUM

Isolate					
None	P6	P7	P9	P10	P11
82.5	6.9	2.5	0	0	12.5

CHAPTER III

CULTURAL STUDIES

From the pathogenicity test described in the previous chapter, three isolates were selected for subsequent experiments, they were P10 (high virulence), P6 (mild virulence), and P11 (low virulence). The purpose of the following tests was to find out the optimum temperature and optimum pH for growth of the selected isolates.

Materials and Methods

(1) Temperature and growth:

The semi-synthetic medium used contained the following components: 17 g agar, 10 g dextrose, 1 g asparagine, 500 mg KH_2PO_4, 250 mg $MgSO_4 \cdot 7H_2O$, 50 ug thiamine hydrochloride, 2.5 ug biotin, 732.5 ug $Fe(NO_3) \cdot 9H_2O$, 439.8 ug $ZnSO_4 \cdot 7H_2O$ and 203.0 ug $MnSO_4 \cdot 4H_2O$ per liter. The pH was adjusted to 6.0. After autoclaving for 20 minutes at 121°C the medium was poured into petri dishes (20 ml in each plate). There were four replicates at each of six temperatures (10°, 15°, 20°, 25°, 30°, and 35°C). A plug 7 mm in diameter from a PDA plate of each isolate was placed in the center of each of the 24 plates. For each isolate 4 plates were placed at each temperature. The radial growth of the colony in each plate was measured after 16, 20, 24, 40, 48 and 60 hr of growth.

(2) pH and growth:

The same semi-synthetic medium was also used in this test except: (a) no agar was added, and (b) the amino acid and sugar solutions were sterilized by passing through sintered glass filters before being added to the basal medium.

The medium was prepared (except for the amino acid and sugar) and divided into five portions each of which was adjusted to a pH of 3.5, 4.5, 5.5, 6.5, or 7.5, respectively. Twenty-five ml of medium was added to each 125-ml Erlenmeyer flask. After autoclaving the flasks of basal medium, required amounts of filter-sterilized amino acid and sugar were added to each flask.

The inoculum was grown on PDA plates and incubated at 25°C. After incubating 3-4 days, plugs 7 mm in diameter were cut from the agar as the inoculum. Each isolate and pH combination had four replicates. Harvests were made at 4, 6, and 8 days after inoculation at 25°C. The final pH of the culture medium was recorded after harvest.

The harvests were made by separating the fungus from the liquid medium on a Buchner funnel and washing the mycelium three times with distilled water, placing the harvest in petri dishes in an 80°C oven overnight and obtaining the net dry weight of the mycelium.

Results

The three isolates had different temperature responses (Figure 1). The optimum temperature for growth of P6 was 25°C, and this isolate was sensitive to a temperature change. The growth of P11 was also best at 25°C but not as sensitive as P6 to other temperatures; this

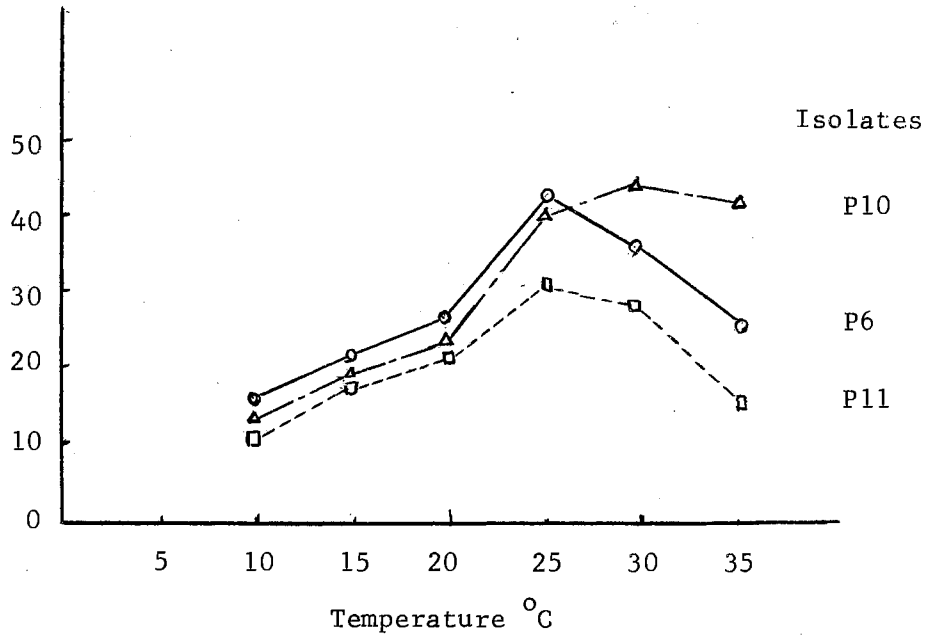

Figure 1. Growth of Three Isolates of Pythium on a Semisynthetic Agar Medium at Different Temperatures After 60 hr.

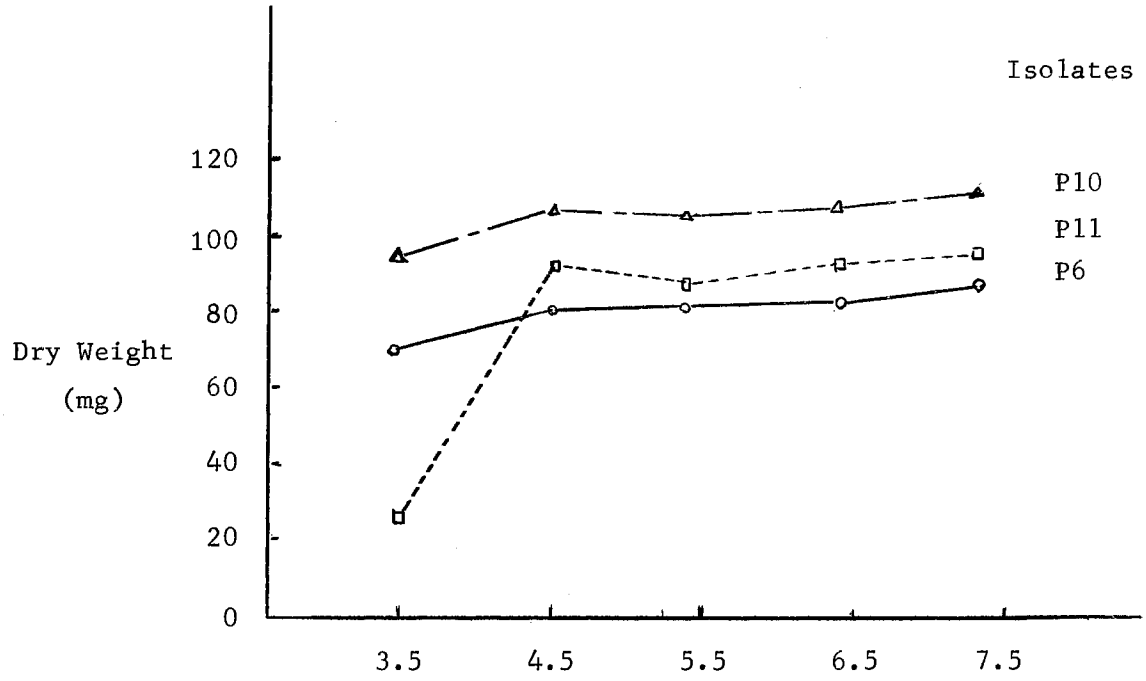

Figure 2. Growth of Three Isolates of Pythium in Synthetic Liquid Medium at Different pH Levels

isolate grew more slowly at the optimal temperature (25°C) than P6 and P10. The isolate P10 grew well over a wide range of temperatures and also was highly virulent; the optimum temperature for growth of this isolate was 25° to 35°C.

All the isolates grew well from pH 4.5 to pH 7.5. Isolate P10 not only grew well over a wide pH range, but also grew well over a wide temperature range. Maximum growth of all isolates was obtained at 6 days.

CHAPTER IV

METHODS OF COLLECTING PLANT EXUDATES, EXTRACTING PLANT TISSUES AND DETERIORATING SEED

Collection of Plant Exudate and Extraction of Plant Tissues

Cotton seed of the Westburn variety was used in the plant exudate and extract experiments. The seeds were surface-disinfested by immersion in a solution containing 10% purex, 10% of 95% ethanol, and 80% water for 3 minutes, after which they were rinsed five times with sterile, deionized water.

White sand, which was washed five times with deionized water was used to collect the exudate. Moist sand was placed in petri dishes of 9-cm diameter (about half-full) and sterilized by autoclaving for 30 min at 15 psi. After the sand cooled, four seeds were planted in each petri dish by pushing the seeds under the surface of sand.

Germinating seeds were harvested at 48 and 72 hr at which time extracts were made and exudates washed from the sand.

Seeds were germinated at two diurnal temperature regimes: (1) 14 hr at 30°C and 10 hr at 20°C and (2) 14 hr at 20°C and 10 hr at 10°C.

Each of the above treatments had four replicates. The method of collecting exudates and extracting seeds is outlined in Figure 3.

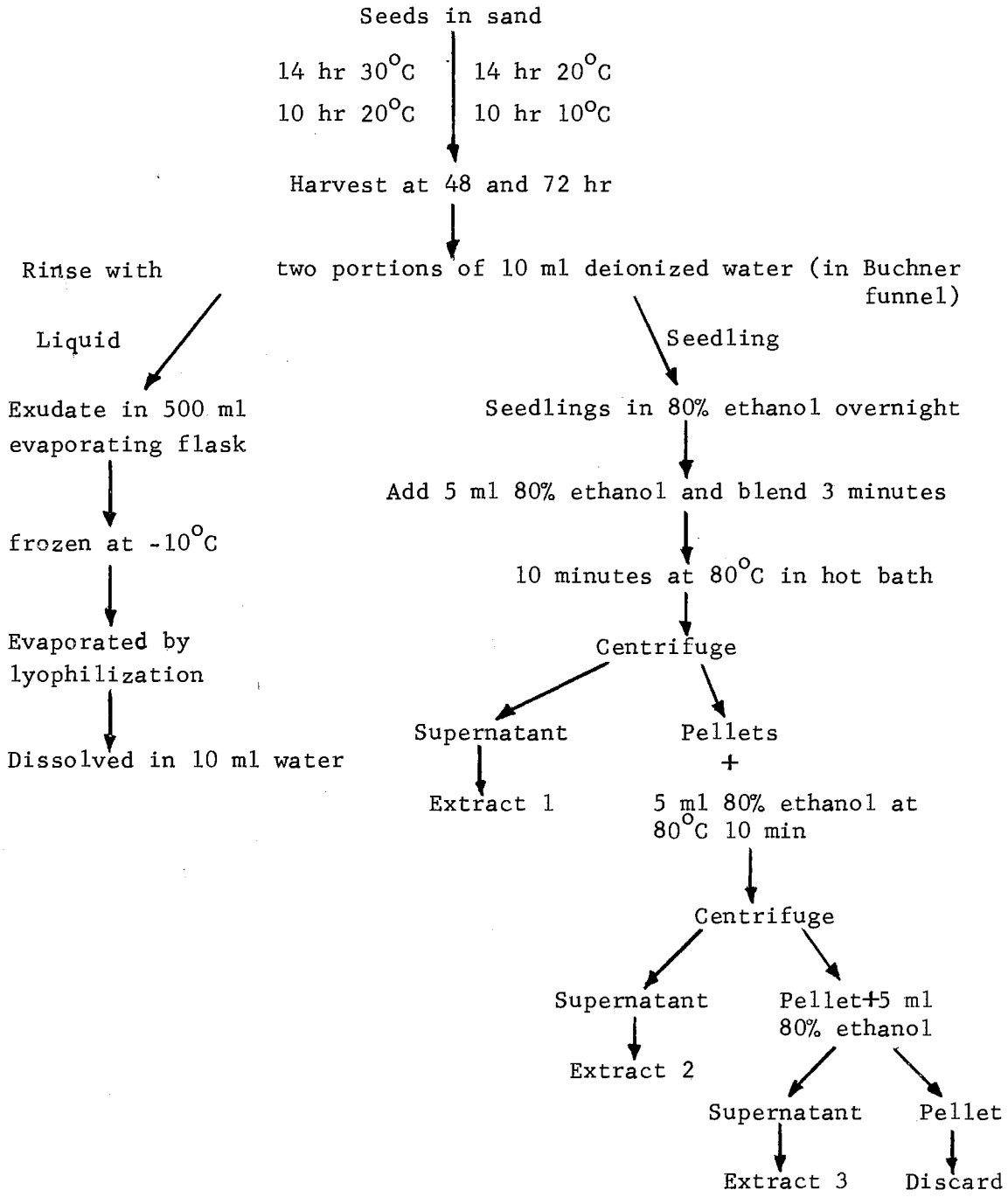

Figure 3. Method of Collecting Exudates and Preparing Plant Extracts

Extracts 1, 2, and 3 from the above steps were pooled (15 ml total) and poured into a 50 ml evaporating flask. The extract was evaporated under vacuum at 45°C until the volume was reduced to 2 ml. The extract was then transferred to 25 ml graduated tubes and the final volume made up to 15 ml with water.

Deterioration of Cotton Seeds

After the cotton seeds were surface-sterilized, they were placed in a quart jar (20 g seed/jar). A 100 ml beaker containing 50 ml water was also placed in each jar, and the jar cap was screwed on tight to keep the humidity at 100%. After incubation 2, 4, 6, and 8 days respectively at 50°C, leachings were obtained by soaking the treated seeds in 100 ml distilled water for 15 minutes at 50°C.

The degree of seed deterioration was indicated by measuring the conductivity of distilled water leachings prepared from the seeds. The increase in leaching is attributed to a loss in the semi-permeable qualities of cell membranes (17). The higher the conductivity the greater is the deterioration. Seeds became progressively more deteriorated with time (Table II).

TABLE II

CONDUCTIVITY OF LEACHINGS FROM SEEDS DETERIORATED FOR VARIOUS PERIODS OF TIME

Length of Treatment	Conductivity (Micromhos)	Resistance (Ohms)
None	4.5	220
2	15	68
4	19	52
6	29	41
8	31	34

CHAPTER V

QUANTITATIVE ANALYSIS OF PLANT EXUDATE AND EXTRACT OF GERMINATING SEEDS

The exudates released from plants may be affected by many environmental factors, among which an important one is temperature. A certain temperature may favor the exudation of amino acids from one plant species, yet with another species the same temperature might cause an increase in the exudation of carbohydrates (49). Several reports have demonstrated that plants release more exudates at low temperatures than at high temperatures. The importance of exudates in influencing severity of seedling disease has been suggested (18, 21, 22, 25, 39, 48, 51). This experiment was designed to study the influence of two temperature regimes (20 D, 10 N, and 30 D, 20 N) on the concentrations of total sugars, amino acids and nitrate ions in both exudates and extracts of germinating seeds, at two growth stages.

Materials and Methods

The exudates and extracts from germinating seeds were prepared according to the procedure described in Chapter IV. These samples were used for the following tests.

(1) Concentration of total sugars

The quantitative determination of total carbohydrates was measured

by Morris' method (36). A Klett-Summerson colorimeter and a K-S filter #54 were used in this test.

Standards used contained glucose at concentrations of 200, 100, and 50 ug/ml.

In this test, 4 ml of sample (or a standard solution) and 8 ml of the reagent (Dreywood's anthrone reagent, 2 g of anthrone dissolved in 1 liter of 95% sulfuric acid) were mixed in 19 mm-test tubes. After 10 to 15 minutes the contents were poured into Klett tubes just before the recordings were made. The color was measured against a blank containing only water and reagent. The color varied directly with the amount of carbohydrate.

(2) Amino acid concentration

The concentration of amino acids was measured by the ninhydrin-positive method (61). The Klett-Summerson colorimeter, with filter #54 was also used in this test.

Reagents

Ninhydrin solution— Dissolve 2 g of ninhydrin (1,2,3-triketo-hydrindene) in 50 ml of fresh methyl cellosolve (2-methoxy-ethanol). Store in refrigerator.

Citrate buffer, 0.5 M— Dissolve 10.5 g of sodium citrate monohydrate in 50 ml of 2N NaOH (8 g/100 ml), adjust pH to 5.0, and make to 100 ml with distilled water.

Standard amino acid solution, 1 mM— Dissolve 75.1 mg of glycine in water and make up to 1 liter.

Stannous chloride-ninhydrin reagent— on the day it is to be used, dissolve 16 mg of $SnCl_2$ in 10 ml of citrate buffer, add 10 ml of ninhydrin solution and mix.

Procedure

Pipette 1 ml of stannous chloride-ninhydrin reagent into each test tube (include a standard and a blank with each test). Add 0.2 ml of sample to 1 ml of reagent in a test tube, mix and cap tube with a clean marble. Place the tubes in boiling water for 20 minutes and add 5 ml of 50% ethanol to each tube after removing from boiling water bath. Mix well and determine absorbance within 15 to 16 minutes after removing from boiling water bath.

(3) Nitrate ion concentration

The concentration of nitrate ion was measured by using the rhenium and α-furildioxime method (7). The same colorimeter and filter were used in this test.

Reagents

α-furildioxime solution— Dissolve 0.35 g of α-furildioxime in 100 ml of methanol.

Stannous chloride, 8.5% — Dissolve 10 g of $SnCl_2 \cdot 2H_2O$ (8.4 g of $SnCl_2$ anhydrous) in 1.0 ml of concentrated HCl and dilute to 100 ml with water.

Potassium perrhenate solution, 0.02%— Dissolve 0.02 g of $KReO_4$ in 50 ml of water and make up to 100 ml with water.

KNO_3 standard— Dissolve 1.63 g of KNO_3 in a total volume of 1 liter of water (1 mg of NO_3^-/ml). When used as a standard solution, dilute to 5-40 ug NO_3^-/ml.

KNO_3 stabilizer solution— Dissolve 2.2 g of KNO_3 in 100 ml of water, and store in refrigerator in dropping bottle (1 mg KNO_3/drop).

Procedure

Add the sample to a 10-ml, graduated test tube and bring total

volume to 2 ml with water. (The sample should contain less than 15 ug to be accurate). To each test tube add 0.2 ml of aqueous 0.02% $KReO_4$ solution, 0.45 ml of concentrated HCl, 3.0 ml of methanol, and 1.0 ml of 8.5% $SnCl_2$. Mix and allow to stand exactly 10 minutes and add 1.0 ml of the α-furildioxime solution. Dilute to 10 ml with water. After exactly 10 minutes, add 1 drop of KNO_3 solution (1 mg KNO_3/ml) and mix. Read the absorbance by K-S colorimeter (filter #54) within one hour.

The intensity of color is inversely proportional to the amount of NO_3^-. Therefore, the blank (darkest) tube was used to set the colorimeter at an arbitrary value of 300.

Results

(1) Concentration of total sugars

After 48 hr and 72 hr, the sugar concentration of the extracts at the low temperature regime was approximately two times greater than at the high temperature regime (Table III). The sugar concentration of exudates was greater at both stages at the low temperature regime than at the high temperature regime. These results with the exudates agree with Rajagopalan and Bhuraneswari (39) who suggested that the first 72 hr of germination appeared to be a critical period in disease development because the exudation increased during this period.

(2) Concentration of amino acid

The concentration of amino acids in both the exudate and extract after 48 hr incubation at the low temperature regime was higher than at the high temperature.

However, after 72 hr, the concentration of amino acids at the

TABLE III

EFFECTS OF TEMPERATURE ON THE CONCENTRATION OF TOTAL SUGARS[1] IN PLANT EXUDATES AND EXTRACTS AT TWO GROWTH STAGES

Temperature regime[4]	Seed stage[2] (ug/mg seed)		Crook stage[3] (ug/mg seed)	
	Exudate	Extract	Exudate	Extract
20 D, 10 N	0.84	33.92	0.54	26.06
30 D, 20 N	0.68	15.92	0.27	15.54

1. Total sugars are expressed as ug equivalents of glucose/mg seed fresh weight.
2. Seeds incubated in sand for 48 hr.
3. Seeds in sand for 72 hr.
4. 20°C days, 14 hr; 10° night, 10 hr.
   30°C days, 14 hr; 20° night, 10 hr.

low temperature regime was less than at the high temperature regime (Table IV).

(3) Concentration of nitrate ion

The concentration of nitrate ion in both extracts and exudates was greater at the low temperature regime than at the high temperature regime, especially after 72 hr (Table V).

TABLE IV

EFFECT OF TEMPERATURE ON AMINO ACID CONCENTRATION[1] IN EXUDATE AND EXTRACT AT TWO GROWTH STAGES

Temperature regime[4]	Seed stage[2] (uM/g seed)		Crook stage[3] (uM/g seed)	
	Exudate	Extract	Exudate	Extract
20 D, 10 N	0.175	4.99	0.307	42.84
30 D, 20 N	0.104	3.57	0.488	48.23

1. Amino acid concentration expressed as uM equivalents of glycine/g seed fresh weight.
2. Seeds incubated in sand for 48 hr.
3. Seeds grown in sand for 72 hr.
4. 20°C day, 14 hr; 10°C night, 10 hr.
   30°C day, 14 hr; 20°C night, 10 hr.

TABLE V

EFFECT OF TEMPERATURE ON NITRATE CONCENTRATION[1] IN EXUDATES AND EXTRACTS AT TWO GROWTH STAGES

Temperature regime[4]	Seed Stage[2] (ug/g seed)		Crook Stage[3] (ug/g seed)	
	Exudate	Extract	Exudate	Extract
20 D, 10 N	310	136.5	312	277.5
30 D, 20 N	210	106.5	81	39.75

1. Nitrate concentration expressed as ug NO_3/g seed; KNO_3 was used as a standard.
2. Seeds incubated in sand for 48 hr.
3. Seeds grown in sand for 72 hr.
4. 20 D, 10 N-20°C day, 14 hr; 10°C night, 10 hr.
   30 D, 20 N-30°C day, 14 hr; 20°C night, 10 hr.

CHAPTER VI

QUALITATIVE STUDIES OF SUGAR CONTENT IN PLANT EXUDATES AND EXTRACTS

Environmental factors affect the quantity of exudates, and may also affect the kinds of compounds exuded. A number of reports (11, 13, 16, 17, 53) have indicated that there are differences in the composition of exudates from resistant and susceptible plants, and also a number of reports (4, 21, 22, 25) have suggested that at low temperatures the susceptibility of plants is increased. The shift of resistance may be related to the pattern of exudates.

The purpose of this experiment was to study the effects of two temperature regimes on the quality of sugar content in exudates and extracts.

Materials and Methods

The sugars were detected by thin-layer chromatography (TLC). The TLC plates were prepared by suspending 15 g of cellulose 300 MN in 90 ml of water-methanol (5:1 v/v). After blending 30 seconds in an Omni-mixer, the mixture was poured into a TLC-spreader and spread 0.37 mm thick on 5 glass plates. After the plates were air-dried, the edges were stripped with a razor blade, and the spotting line (1.5 cm from the bottom edge of the adsorbent) and solvent-stopping line (15 cm from spotting line) were marked with a pencil. The plates were

then placed in an oven at 105°C for 10 minutes, after which time they were stored in a desiccator.

The sample was spotted on the plate by using a micro-syringe. For each series of samples, two plates were spotted. One plate was sprayed with reagent A, and the other plate with reagent B.

The separation solvent was composed of:

Formic acid	30 ml
Methyl-ethyl ketone (2-butanone)	60 ml
tert-Butanol	80 ml
Water	30 ml

The solvent was placed in a separation tank at least 15 minutes to obtain a saturated condition in the tank atmosphere before adding the plates. The plates were run twice for a distance of 15 cm and air dried after each run.

Two spray reagents were used.

1. Spray reagent A, contained:

p-Anisidine	1.23 g
Phthalic acid	1.66 g
95% Ethanol	100 ml

After spraying, the plates were heated at 120°C for 10-12 minutes.

2. Spray reagent B, contained:

Thymol	0.5 g
Concentrated H_2SO_4	5 ml
100% Ethanol	95 ml

After spraying, the plates were heated at 120°C for 5-7 minutes. The sugars reacting with reagent A produced brown spots and the sugars reacting with reagent B produced grey or pink spots. Fructose reacted with both reagents A and B. Fructose was chosen as the standard.

Results

Stachyose, maltose, galactose, and glucose reacted with spray reagent A, but not with reagent B; raffinose, melibiose, and sucrose reacted with spray reagent B, but not with reagent A; fructose reacted with both reagents.

The TLC tests showed little qualitative difference between the sugar contents from the low temperature and the high temperature regimes for any given time period (Figures 4 and 5), but there were differences between exudates and extracts.

Maltose and sucrose were present at all stages in both exudates and extracts; galactose was found in exudates, but not in extracts. Raffinose was found both in the exudates and extracts; stachyose was found in the extracts, but not in the exudates.

Glucose was found in all the exudates and in the 72-hr extracts, but was not detected in the 48-hr extracts at both the high and low temperature regimes; however, in these latter two samples some type of pentose (possibly ribose) was detected.

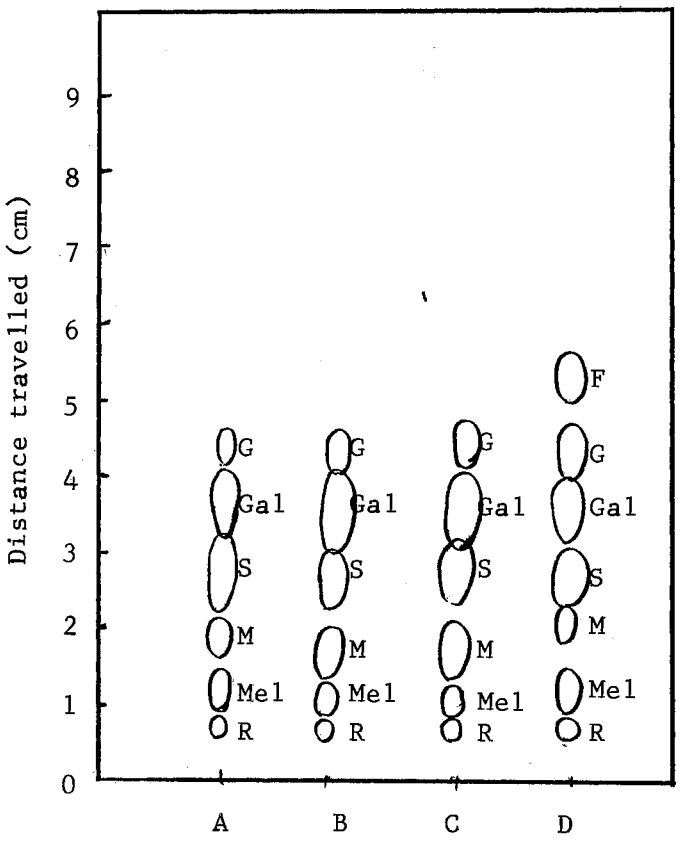

Figure 4. Sugars Present in Exudates

A, 20 D, 10 N, 48 hr; B, 30 D, 20 N, 48 hr; C, 20 D, 10 N, 72 hr; D, 30 D, 20 N, 72 hr; G, glucose; Gal, galactose; M, maltose; Mel, melibiose; R, raffinose; S, sucrose; F, fructose.

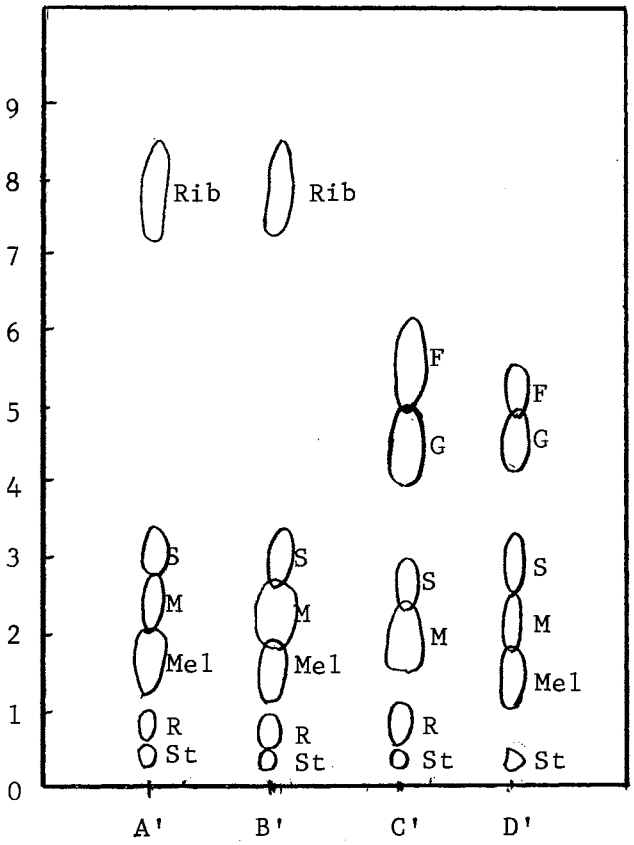

Figure 5. Sugars Present in Extracts

A', 20 D, 10 N, 48 hr; B', 20 D, 20 N, 48 hr; C', 20 D, 10 N, 72 hr; D', 30 D, 20 N, 72 hr; F, fructose; G, glucose; M, maltose; Mel, melibiose; R, raffinose; Rib, ribose; S, sucrose; St, stachyose

CHAPTER VII

THE EFFECT OF PLANT EXUDATES ON GROWTH OF *PYTHIUM* AND ON DISEASE DEVELOPMENT

Growth of *Pythium* in Exudate

One of the major factors determining the behaviour of plant pathogens in soil is the availability of nutrients. An important source of nutrients in the rhizosphere is exudation from plant roots. Growth of pathogens in exudates should be proportional to the concentration of nutrients in the exudates. The purpose of this test was to determine if exudate from the low temperature treatment would support more growth of *Pythium* than exudate from the high temperature treatment.

Materials and Methods

Exudates from the low temperature regime (20 D, 10 N) and from the high temperature regime (30 D, 20 N) were used in this test. The exudates were sterilized by passing through sintered glass filters after adjusting the concentration according to the plant weight at the two temperature regimes. Twenty-five ml of exudate solution was added to each 125-ml Erlenmeyer flask.

The inoculum was grown on PDA plates and incubated at 25°C. After incubating 3-4 days, plugs 7 mm in diameter were cut from

the agar as the inoculum. Twelve replicates were used. The harvest was made after incubating 6 days at 25°C by separating the fungus from the liquid medium on a Buchner funnel and washing the mycelium three times with distilled water, placing the harvest in petri dishes in a 80°C oven overnight and obtaining the net dry weight of the mycelium.

Results

The dry weight of mycelium grown in different exudates were as follows:

Temperature regime of exudate source	Mycelium dry weight (mg)
20 D, 10 N	56.3
30 D, 20 N	45.7

Fungal dry weight of Pythium grown in exudate from the low temperature treatment was about 23% heavier than grown in exudate from the high temperature treatment after 6 day's incubation. This means that exudates from the low temperature regime contained a higher concentration of nutrients that supported growth of Pythium than did exudates from the high temperature regime.

Effect of Exudates on Disease Development

Many reports (18, 20, 25, 52, 57) have indicated that exudation was higher at low temperatures than at high temperatures and a number of reports (4, 17, 18, 19, 22, 50, 51) have also indicated that root-rot or seedling diseases were more severe at low temperatures than that at high temperatures. If the exudates were the major factor

contributing to disease development, then disease development should be increased, if exudate from the low temperature regime were added to inoculated plants grown at the high temperature regime in soil.

In these experiments, pots of soil were planted with seed and infected with Pythium. The exudates, or pure chemicals representing exudates, added to the pots, were as follows:

(1) Exudate from seeds germinated at the low temperature regime or pure chemicals representing exudates or extracts (synthetic exudate or extract).

(2) Concentrated exudate from seeds germinated at the low temperature regime.

(3) Concentrated exudate from deteriorated seeds germinated at the low temperature regime.

Materials and Methods

The collection of exudate was described in Chapter IV. The exudate for each petri dish which contained 10 seeds was added to each pot. Seeds were germinated at the low temperature regime (20 D, 10 N), and exudates were washed twice at 48 hr and 72 hr from the sand. The exudate collected at 48-hr was added at the time of inoculation; that at 72-hr was added 48 hr after inoculation.

In the second test the exudates were concentrated to six times that of the above exudates by low temperature evaporation.

When exudate was collected from deteriorated seeds, those seeds deteriorated for 4 days (Chapter IV) were used. This exudate was also concentrated to six times the original concentration.

The amount of pure chemicals (synthetic exudate or extract) added

was at the rates found by subtracting the concentrations in exudates and extracts of germinating seeds at the high temperature regime from those at the low temperature regime. The amounts of sugar (glucose) and amino acid (glycine) added are listed in Table VI.

The inoculation method was almost the same as used in the pathogenicity test (Chapter II). Before inoculation, the soil was first moistened and then 50 ml of inoculum was added evenly over the soil surface. The inoculum was then covered with a thin layer of soil and the seeds planted on the soil surface. Finally, 40 ml of exudate (or chemical mixture) were added and the pot covered with plastic which was held tight with a rubber band. The planted pots were kept in a growth chamber at a temperature regime of 30 D, 20 N. After 48 hr, the same amount of exudate was again added and then covered with 2-3 cm of soil. Seed germination was calculated 2 weeks after planting, and roots were examined for infection after the final count.

Results

In the first experiment, in which plant exudates or synthetic exudates or extracts were added to pots of soil containing cotton seed and Pythium inoculum, disease development was not affected (Table VII). Possible explanations for these results are:

(1) The chemicals or exudates added had no influence on disease development, or the concentration of exudate was not high enough.

(2) Isolate P10 was a highly virulent isolate and the inoculum concentration used may have masked the effect of exudates on disease development.

(3) The nutrient content of the inoculum (prepared from a PDA plate) may have been high enough to mask the effect of nutrients added

TABLE VI

THE COMPOSITION OF SYNTHETIC EXUDATES AND EXTRACTS USED IN THE TEST REPORTED IN TABLE VII

Composition	Synthetic Exudate[1]		Synthetic Extract[1]	
	A	B	A	B
Glucose (mg/40 ml)	1.5	5.7	51.9	217.3
Glycine (μM/40 ml)	1.25	1.26	2.3	2.3

[1]The synthetic exudates and extracts contained concentrations of glucose and glycine which were equivalent to total sugars and amino acids obtained from seedlings germinating at a temperature regime of 20°C day and 10°C night; A represents the concentrations found after 48 hr germination and B after 72 hr germination.

TABLE VII

EFFECT OF ADDING SYNTHETIC EXUDATES, SYNTHETIC EXTRACTS, OR PLANT EXUDATES ON PERCENT OF SURVIVING COTTON SEEDLINGS IN SOIL INFESTED WITH PYTHIUM SP.

Inoculum[1]	Percent Surviving Seedlings[2] Treatment[3]			
	Synthetic exudate	Synthetic extract	Plant exudate	Water
P10	26.7	30.0	28.3	27.6
CK	100	95.0	96.7	98.3

1. Inoculum was grown on PDA plates. Each plate was blended with 150 ml water and 50 ml was added to each pot.

2. Percent based on number of seed planted.

3. This test was conducted in a growth chamber set at 30°C days (14 hr) and 20°C nights (10 hr). The plant exudate was collected from seeds germinated at 20°C days and 10°C night. The synthetic exudate and extract contained glucose and glycine concentrations equivalent to sugars and amino acids found in actual exudates and extracts from seeds germinated at 20°C days and 10°C nights.

in the exudate.

To eliminate most of the above possibilities, the next inoculation test included (i) two isolates, P10 and P6, (ii) inocula grown in liquid medium, and (iii) exudates concentrated six times. In the second inoculation test, disease development appeared to increase when concentrated exudates were added to the soil in the presence of either isolate P10 or P6 (Table VIII). However, the increase in disease is not considered real because the variation existing within each replicate precluded the existence of statistical significance between means.

In the third inoculation test, the addition of exudate from deteriorated seeds did not influence healthy seed germination but did slightly decrease disease development, although probably not significantly.

It is possible that the exudates added here had no effect on disease development because of the concentration of exudates, composition of exudates, or the exudate was contaminated with bacteria which could have behaved as antagonistic microorganisms. Henis and Inbar (24) suggested that Bacillus subtilis (a heat-stable bacterium) inhibited the growth of Pythium sp. This bacterium exists abundantly in air and soil.

TABLE VIII

EFFECT OF CONCENTRATED PLANT EXUDATES ON PERCENT OF SURVIVING COTTON SEEDLINGS IN SOIL INFESTED WITH PYTHIUM SP.

Inoculum[1]	Percent Surviving Seedlings[2] Treatment[3]	
	Exudate Added	Water
P10	21.3	30.0
P6	55.0	61.7
CK	95.3	96.7

1. Inoculum was grown 4 days in 50 ml liquid medium, blended in 150 ml H_2O/flask, and 50 ml added/pot.

2. Percent based on number of seed planted.

3. This test was conducted in a growth chamber set at 30°C days (14 hr) and 20°C nights (10 hr). The plant exudate was collected from seeds germinated at 20°C days and 10°C nights and then concentrated 6x.

TABLE IX

EFFECT OF CONCENTRATED EXUDATE FROM DETERIORATED SEEDS ON PERCENT OF SURVIVING COTTON SEEDLING IN SOIL INFESTED WITH PYTHIUM SP.

Inoculum[1]	Percent Surviving Seedlings[2] Treatment[3]	
	Exudate Added	Water
P10	51.0	42.7
P6	67.3	56.6
CK	96.7	98.5

1. Inoculum was grown 4 days in 50 ml liquid medium, separated from medium and blended in 150 ml H_2O/flask, 50 ml added/pot.

2. Percent based on number of seed planted.

3. This test was conducted in a growth chamber set at 30°C days (14 hr) and 20°C nights (10 hr). The plant exudate was collected from deteriorated seeds germinated at 20°C days and 10°C nights and then concentrated 6x.

CHAPTER VIII

DISCUSSION AND CONCLUSION

It was found that a trapping technique using eggplant fruits was the best method of isolating Pythium from infected cotton seeds.

That root exudation increases at low temperatures has been suggested by many reports (15, 20, 21, 22, 23, 26, 31, 33, 51). In this study (Chapter IV), I found that the concentrations of total sugars, amino acids and nitrate ion in exudates generally increased at a low temperature regime except that the amino acid concentration in exudates at the low temperature regime decreased after the seeds had germinated for 48 hours. Buxton (13) and Rajagopalon and Bluvaneswari (39) suggested that exudate is produced abundantly during the first 72 hr of seed germination (39), and then proportionally less as the seeds develop (47, 49).

Extracts of seeds germinated at low temperatures also contained greater concentrations of sugars, amino acids, and nitrate ion than did those from seeds germinated at high temperatures.

From the thin-layer chromatography of sugars I found that the temperature regime had no effect on the kinds of sugars found either in exudates or in extracts. The temperature may effect the rate of metabolism, but probably does not affect the pathway of metabolism. Shiroya (53) found that stachyose was found only in cotton seeds and was formed from raffinose by transgalactosidation; these two

oligosaccharides decreased after seed germination at which time a considerable amount of sucrose was found. In my study stachyose was found only in extract and not in exudate. Shiroya also showed that no galactose was detected through all stages of germination. I found (Chapter VII) that galactose was detected in the exudate but not in the extract. Shiroya found that glucose existed in all stages of germinating seed, but I found no glucose in the extract of 48 hr samples. It is possible that all of the glucose in the seeds was used as an energy source under the high rate of metabolism. A number of reports have indicated that there are differences in the composition of exudates from resistant and from susceptible plants (11, 13, 55). Flentje (16, 18) found that sucrose was a major factor governing susceptibility of peas to pre-emergence damping-off, and he also found that when he added the exudates from susceptible seeds to soils in which resistant seeds were planted, the resistance was broken down. However, Rovira (47) suggested that the quantity of exudate was more important than the composition of exudate in influencing the incidence of disease.

Plant exudates may directly or indirectly affect a pathogen by inducing spore germination, and stimulating mycelial growth. It was found that the dry mycelial weight of Pythium sp. grown in the exudate from the lower temperature regime was twenty percent heavier than the mycelium grown in the exudate from the higher temperature regime. This means that the exudate contained nutrients needed for growth of the pathogen, and that the low temperature regime produced more of these nutrients than the high temperature regime. The sugar concentration in the exudates at the low temperature regime was

nearly 20% higher than that at the high temperature regime which could account for all of the increase in growth of the fungus.

According to several reports increased incidence of root diseases are related to increased materials in the exudate (11, 13, 16, 18, 20, 39, 48, 51). If the increase in disease is due only to increased exudation then adding exudate from the low temperature regime to plants growing at the high temperature regime should cause an increase in disease. This hypothesis was not substantiated by my studies.

In the first inoculation test (Chapter VII) disease incidence was not increased by adding exudates or pure chemicals. Several possibilities were discussed in Chapter VII as to why the additions might not have had any effect on disease development. The second inoculation test was designed to eliminate most of these possibilities. In the second inoculation test with concentrated exudates, I found that the disease increased when the exudate was added to soil, but the evidence was not convincing because of variation between replicates.

Presley (38) suggested that deteriorated cotton seeds are more susceptible to seedling disease than high-quality seeds. Hunter (unpublished data) found that the viability of high-quality seed was decreased by adding the leachate from deteriorated seeds. However, in my experiment with exudates from deteriorated seeds the exudates had no effect on either seed germination or disease incidence.

Although exudates may stimulate spore germination of certain pathogens and they may also add to their growth, it is not necessarily a corollary that pathogen virulence is increased. The constituents and concentration of host exudates required to influence spore germination and growth may or may not be optimal for production of

toxins, or production of other metabolites which contribute to the virulence of an organism. Successful infection by an organism also depends on many other environmental factors during the process of infection.

It has been shown for some fungi, that they require a certain level of nutrients in the environment before infection (26, 59) and hence the nutritional status of the inoculum may govern the possibility of infection. On the other hand, exudate may also decrease the inoculum potential by the presence of toxic materials such as HCN and glycosides, which inhibit the growth of pathogens; and by increasing the activities of antagonistic (12) or competitive microorganisms. Buxton (12) has suggested that under natural conditions, root exudates are unlikely to be the dominant factor in the rhizosphere because the microorganisms rapidly change the chemical nature of the exudates. Rovira (46) also showed that the amino acid patterns in exudates were altered by the presence of microorganisms, probably owing to the utilization of certain amino acids and the release of others by the microorganisms.

In conclusion, it can be said that there is little doubt that root exudates play an important role in the establishment and maintenance of populations of microorganisms in the rhizosphere of the seedling; however, their effect on the virulence of microorganisms has not been conclusively demonstrated.

The result of my tests suggest the possibility that although increased exudation from plant roots at low temperatures is related to increased disease incidence, the increased exudation may not be the cause of increased disease incidence. There is a good possibility

that increased exudation is merely coincidental to a loss of resistance in seedlings grown at low temperatures, and that the important effect of low temperatures on plants is a loss of seedling disease resistance rather than an increased growth of the pathogen due to the increased root exudation.

CHAPTER IX

SUMMARY

The use of eggplant fruits to isolate Pythium from soil or seeds was found to be the best isolation method. The virulence of isolates obtained by this method ranged from high to low.

The optimal temperature for growth of the isolates tested was $25^{\circ}C$-$30^{\circ}C$. All isolates grew well over a pH range of 4.5-7.5.

The concentration of sugars in exudates and extracts of germinating cotton seed was higher at a low temperature regime (20 D, 10 N) than at a high temperature regime (30 D, 20 N). The sugar concentration in the exudate from the low temperature regime was 20% higher than from the high temperature regime after 48 hr, and 50% higher after 72 hr. The amino acid concentration in the exudate and the extract at the low temperature regime was also higher than at the high temperature regime after 48 hr, but the exudate and extract after 72 hr was higher at the high temperature regime than at the low temperature regime. The nitrate ion concentration was higher at the low temperature regime than at the high temperature regime; especially after 72 hr. The qualitative analysis of sugars showed no difference beeween extracts from the low temperature and high temperature regimes; however, there were differences between exudates and extracts. Maltose and sucrose were present at both 48 and 72 hr in both the exudate and the extract; galactose was found only in the exudate,

but not in the extract. Raffinose was found both in the exudate and the extract. Stachyose was found in the extract, but not in the exudate. Glucose, a very common sugar, was not detected in the extract of the 48-hr samples either at the high temperature or at the low temperature regime. Some type of pentose was detected in these two samples.

The dry weight of Pythium grown in exudate from the low temperature treatment was 23% heavier than when grown in exudate from the high temperature treatment. This means that the exudates from the low temperature regime contained a higher concentration of nutrients that stimulated growth of the isolates of Pythium sp. used in these studies than did the exudates from the high temperature regime.

When the exudates collected from germinating cotton seeds grown under the low temperature regime were added to Pythium infested soil incubated under the high temperature regime, no marked effect on cotton seedling disease was demonstrated. It is suggested that, increased exudation at low temperatures may not be the cause of increased disease incidence, but merely related to it.

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