# THE EFFECT OF TEMPERATURE ON THE CHEMICAL COMPOSITION OF HYPOCOTYLS OF COTTON AND ON THE SEEDLING DISEASE INCITED BY RHIZOCTONIA SOLANI

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## PREFACE

Seedling diseases are responsible for at least an estimated 3% reduction in yield of cotton (Gossypium hirsutum L.) each year in Oklahoma. This accounts for 1/4 to 1/3 of that portion of the cotton crop which is lost to diseases. The use of fungicides, high-quality seed, and improved machinery help to reduce these losses but do not give satisfactory control under adverse environmental conditions.

Other possible approaches to the control of seedling diseases of cotton include breeding for resistance and modification of the plant's metabolism by the addition of chemicals. The success of both of these measures would be hastened by knowledge of the host's metabolism and chemical composition under environments which alter the host's resistance or susceptibility. This dissertation adds to basic knowledge of the effect of environment on the chemical composition of the cotton plant and on the susceptibility of cotton to the seedling disease incited by Rhizoctonia solani Kuehn.

I would like to express my appreciation for the guidance given me by members of my committee: Dr. L. A. Brinkerhoff, for his constant encouragement and friendly help in many ways; Dr. Gene Guinn, who suggested the problem and supplied much valuable information; Dr. John Thomas, Dr. Eddie Basler, Dr. George Waller and Dr. Glen Todd for their continued interest and counsel. I also gratefully acknowledge the encouragement and counsel of Dr. W. W. Hansen, deceased.

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I am grateful to Dr. J. T. Presley for the opportunity to conduct these studies while employed by the United States Department of Agriculture and also for his personal encouragement.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

## INTRODUCTION

*Rhizoctonia solani* Kuehn, *Pythium* spp. and *Fusarium* spp., are the most important organisms in the seedling disease complex of cotton in Oklahoma. Ray and McLaughlin (38) considered *R. solani* to be of primary importance because of its high degree of virulence and its frequency in Oklahoma soils. They also found several species of *Fusaria* to be involved in the seedling disease complex. Subsequent isolations and pathogenicity tests by Brinkerhoff and Hunter (unpublished) have shown *Pythium* spp., as well as *R. solani* and *Fusarium* spp., to be important seedling disease organisms. *R. solani* was chosen for the work reported in this dissertation.

Seedling diseases of cotton are generally more severe during cool, wet weather than during warm, dry weather. It has been demonstrated that *R. solani* (2, 27, 39, and 46), *Fusarium* spp. (2) and *Pythium* spp. (2, 27) cause more damage at temperatures below 25°C than above. However Hunter *et al.* (25) and Maier and Staffeldt (31) obtained isolates of *R. solani* which caused damage over a wide range of temperatures (20-30°C) and other workers (41, 45) reported that disease severity was greatest at temperatures above 25°C. Maier and Staffeldt (31) found that 8 of the 12 isolates they worked with were temperature independent but the other 4 produced more injury at the lower temperatures.

Several factors affect the relationship between temperature and

disease incidence under field conditions. Garrett (17) pointed out that temperatures affect antagonistic organisms as well as pathogens. Temperature also affects biochemical composition of the host. Recent reviews (1,6,43,48) have covered the physiology and biochemistry of the plant in relation to disease resistance or susceptibility.

An increase in sugar content of the host sometimes favors development of the pathogen (22). The content of total sugars in plants generally increases as temperature decreases (18,19,26,36). The same tendency has been found also for sucrose and reducing sugars (4,11,18, 19,42). This is partly the result of starch to sugar conversion and partly the result of a greater reduction of respiration than photosynthesis at low temperatures (32). Physiological drought induced by low root temperatures may also promote carbohydrate accumulations in cotton. Eaton and Ergle (14) reported that carbohydrates accumulated in cotton at low moisture levels despite the fact that drought slows photosynthesis. Guinn and Hunter (19) found that sugar content of cotton seedlings, expecially in the stem and upper tap roots, increased greatly as the temperature was lowered. Stem homogenate from chilled seedlings supported more growth of R. solani in culture than did stem homogenate from unchilled seedlings (19).

Lewis (29) proposed that many host-parasite relationships might be explained in terms of metabolites of the host and nutrition of the parasite. Garber (16) demonstrated the importance of concentration of host metabolites at the site of entry of the pathogen. Weinhold and Bowman (47) suggested that available organic nutrients are an important factor affecting the virulence of R. solani towards cotton. Nezgovorov (34) showed that the effect of low temperature on increased incidence

of the seedling disease caused by Pythium spp. 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. Low temperatures also increased production of toxins by the fungus and decreased the activity of antagonistic organisms in the soil. Blackmon (10) found that cotton plants which were susceptible to bacterial blight contained more ninhydrin-reacting materials than those which were not susceptible.

The mineral-nutrient status of the host also affects disease susceptibility. Castano and Kernkamp (12) demonstrated that soybeans which were grown in calcium-deficient nutrient cultures had a cellular structure which allowed for easy penetration and invasion by R. solani. Zyngas (49) studied the effect of inorganic ions on seedling disease of cotton incited by R. solani and was able to partially relate the effects of some ions on disease expression to structure of host cells. Bateman (7) found that replacing exchangeable cations with Ca in excised bean hypocotyls made the tissues more resistant to attack by R. solani and related the resistance to accumulation of calcium pectates which are resistant to the action of polygalacturonase produced by the fungus. Bateman and Lumsden (8) found that the resistance of bean hypocotyl tissue increased with age and was associated with the change of pectins to calcium pectate.

More recently, Pierre and Bateman (37) found phytoalexins were produced in infected bean tissues and these appeared to be an important factor in limiting the size of lesions due to R. solani. Cruikshank (13) reviewed the literature on phytoalexins up to 1963. Bell (9) proposed that excised stems and bolls of cotton plants produce free gossy-

pol (not contained in the glands) in response to disease organisms and other stimuli, and that this substance acts as a phytoalexin.

Kuc (28) outlined the possible mechanisms of disease resistance as follows:

A. Physical or chemical barriers

1. Lack of necessary growth factors

2. Preformed inhibitors of microbial growth

3. Detoxification of enzymes or toxins

B. Physiological stress

Production, liberation or mobilization of compounds following infection or injury

C. Unmasking of host DNA

Synthesis of specific RNA resulting in control of metabolic function through enzyme synthesis.

No definite type of resistance to seedling disease has been found in the cotton plant. On the contrary, the seedling seems to vacillate between a state of susceptibility and resistance depending on the environment. Rubin and Artsikhovskaya (40) in discussing the significance of the chemical composition of the plant in relation to disease, have, in effect, restated A 1 and 2 in the above outline in a form more pertinent to the present investigations as follows:

> Susceptibility...depends on whether plant tissues are rich in substances essential for the nutrition of the parasitic microorganisms. Resistance, on the other hand, is determined either by a deficiency in the plant of such substances, or by the presence in the cells of the plant host of compounds toxic for the pathogen.

This quotation essentially describes the nature of the investigations that were made for this dissertation with the additional consideration that the chemical composition of the plant is influenced by environ-

ment. The environmental factor studied most thoroughly was temperature; limited studies were also made on age of plant and nutrient levels.

# CHAPTER II

# TEMPERATURE STUDIES IN GROWTH CHAMBERS

## Nutrient Solutions

### Methods and Materials

Acid-delinted, Panogen-treated cotton seed (Gossypium hirsutum, cv Stoneville 62) were planted in vermiculite and held at 25°C until the cotyledons had expanded (usually 5 days). The seedlings were then transferred to a modified Hoagland's (3) solution (Table I). The plants were held between 2 redwood strips lined with polyurethane. Two sets of strips were placed in slots in a wooden cover over each container of solution (24 and Appendix A). Twenty plants per container served as one treatment and each treatment was replicated four times.

After the plants were transferred to the solution they were placed in a growth chamber where the light intensity during a 14-hr. light period was 2500 ft-c at the tops of the containers. The light source had the approximate ratio of five w supplied by cool-white, fluorescent tubes to each w supplied by incandescent bulbs. The seedlings were held at 25°C for 1 week after which they were subjected to different temperature regimes. Plants to be harvested and analyzed for sugars and ninhydrin-positive (Nh-pos) substances were either left at 25°C or moved to a chamber at 15°C and harvested at 0, 2, and 4 days during the period of the experiment. Plants to be inoculated with R. solani were placed at several temperature regimes described below.

TABLE I

MODIFIED HOAGLAND'S SOLUTION USED FOR GROWING COTTON PLANTS

| Chemical | Concentration (mg/liter) |
|---|---|
| $Ca(NO_3)_2 \cdot 4H_2O$ | 472.00 |
| $MgSO_4 \cdot 7H_2O$ | 246.00 |
| $KNO_3$ | 202.00 |
| $KH_2PO_4$ | 68.00 |
| $H_3BO_3$ | 2.40 |
| $MnSO_4 \cdot H_2O$ | 1.20 |
| $ZnSO_4 \cdot 7H_2O$ | 0.36 |
| $CuSO_4 \cdot 5H_2O$ | 0.03 |
| $CoCl_2 \cdot 6H_2O$ | 0.06 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.20 |
| Fe-HEEDTA | (2 ppm Fe) |
| $H_2SO_4$(conc.) | (0.048 ml) |

The pH of the nutrient solution made up in tap water was approximately 6.

All chemicals except the Fe-HEEDTA were reagent grade.

As the plants were harvested, they were washed in tap water and the tops cut off at the cotyledonary node. The secondary roots and the ends of the tap roots were removed and the remaining portions of the hypocotyls were placed in plastic bags at 4°C.

After all the plants were harvested, plant extracts were prepared. The contents from one plastic bag (20 hypocotyls) were rinsed in deionized water and then homogenized in a Servall Omni-mixer with 100 ml of water for 3 minutes at top speed. Water was added to the resulting homogenate to make a total mixture weighing 200 g which was centrifuged at 12,000 x g for 15 minutes. The supernatant was stored at -14°C until needed.

Plant extracts to be used for the determination of Nh-pos substances were clarified by adding 0.2 ml of a saturated lead acetate solution to 5 ml of the thawed supernatant. This was mixed and allowed to stand 10 minutes. Then 0.2 ml of a saturated solution of $K_2HPO_4$ was added, the solution mixed, and let stand 10 minutes. This mixture was centrifuged and the supernatant used in the determinations. For the sugar determinations, extracts were cleared by the addition of equal volumes of 95% ethanol and centrifuging after 10 minutes.

Reducing and total sugars were estimated by the ferricyanide method of Park and Johnson (35). Hydrolysis for the total sugar tests was accomplished by adding an equal volume of 1N $H_2SO_4$ to the plant extract and heating in boiling water for 30 minutes. Nh-pos substances were determined by the ninhydrin method of Moore and Stein (33) as modified by Fels and Veatch (15).

Inoculation of plants growing in the containers of nutrient solution was made by two methods. One method was to add to each container

100 ml of a suspension prepared from R. solani cultures. The suspension was prepared by blending with water for 1 minute cultures of the fungus which had been grown in Petri dishes at 27°C for 8 days on potato-dextrose agar (PDA). The contents of one Petri dish were contained in 100 ml of suspension. The inoculum was added to the nutrient solutions one week after the plants had been transferred and the disease readings were made 21 days after inoculation. Temperature regimes imposed on the plants after inoculation by this method were 30°C day, 15° C night, and 25°C day, 10°C night.

In the second method inoculations were made by placing the tip of a one-ml pipette between the polyurethane strips and depositing 0.1 ml of the above fungal suspension on each plant hypocotyl. Disease readings were made 8 days after inoculation. Temperature regimes used here were 30°C day, 20°C night and 20°C day, 10°C night.

The disease grades ranged from 0 to 4. Plants with no sign of infection on the hypocotyls were graded 0, while dead plants were graded 4. Grades 1, 2, and 3 were assigned to mildly, moderately, and severely infected plants, respectively (Figure 1). A mean disease index was then calculated for each temperature treatment.

Results

The concentration of total sugars in hypocotyls decreased during the 4-day period at 25°C but increased at 15°C (Table II). These results confirmed earlier evidence that the concentration of sugars was higher in plants grown at low temperatures compared to plants grown at higher temperatures. There was only a small change in the concentration of amino acids, decreasing at 25°C and increasing at 15°C.

Disease expression was slight and equal at both temperature re-

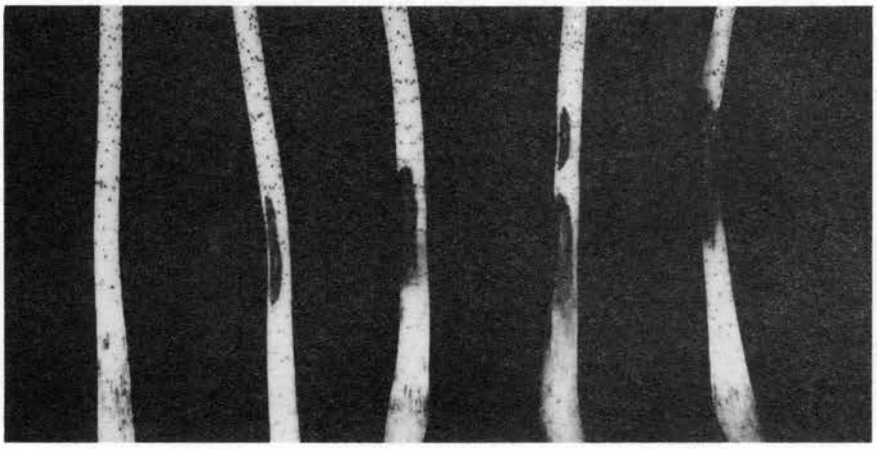

Figure 1. Seedling Disease Grading Scale From Left to Right: 0, 1, 2, 3, and 4.

TABLE II

COMPOSITION OF HYPOCOTYLS GROWN AT 25° OR 15°C IN NUTRIENT SOLUTIONS IN GROWTH CHAMBERS [1]

| | Days at 25°C | | | Days at 15°C | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 0 | 2 | 4 |
| Plant dry weight (mg) | 845 | 963 | 1120 | 920 | 1050 | 1267 |
| Sugar (mg/g plant) | | | | | | |
| Reducing | 12.3 | 12.3 | 10.3 | 23.7 | 22.6 | 29.5 |
| Total | 21.5 | 20.5 | 12.4 | 40.7 | 60.2 | 90.8 |
| Nh-pos substances | | | | | | |
| (μmoles/mg plant) | 0.14 | 0.12 | 0.09 | 0.15 | 0.16 | 0.17 |

[1]Values for sugars and Nh-pos substances are reported on a dry weight basis.

gimes when the inoculum was added directly to the nutrient solutions. When hypocotyls only were inoculated the mean disease indices at the two temperature regimes were as follows:

| Temperature regime | Mean disease index |
|---|---|
| 30 D, 20 N | 1.3 |
| 20 D, 10 N | 2.2 |

The increased disease at the lower temperature regime, compared to disease at the higher regime, substantiated field observations.

## Soil

Another series of tests were set up in growth chambers to determine effects of temperature on composition of the hypocotyl, expression of seedling disease, and in vitro growth of the fungus. This series of tests was somewhat similar to those just described except that pots of soil were used instead of liquid nutrient solutions.

### Methods and Materials

Seeds were planted in 6-inch clay pots containing sandy-loam soil which had been steamed for 14 hours. The pots were set in a growth chamber at 30°C until the seedlings had emerged at which time one of the three following temperature regimes was begun: 30°C day, 20°C night; 25°C day, 15°C night; and 20°C day, 10°C night.

Before the seeds were planted, inoculum of R. solani was added to some of the pots by pouring 20 ml of the fungal suspension (prepared as previously described) on the soil surface below the level of the seeds. A range of inoculum concentrations was used (see Table III). Survival counts were made after 21 days and expressed as the percent of seed planted.

TABLE III

RESULTS OF INOCULATION TESTS USING POTS OF SOIL IN GROWTH CHAMBERS

| Inoculum level[1] | Survival as percent of seed planted | | |
|---|---|---|---|
| | Temperature regime | | |
| | 20 D, 10 N | 25 D, 15 N | 30 D, 10 N |
| 1/25 | 22 | 65 | 0 |
| 1/50 | 45 | 88 | 1 |
| 1/150 | 85 | 95 | 70 |

[1]The inoculum was prepared by blending with 100 ml of water, the contents of one Petri-dish containing a culture of the fungus and PDA. The fungal suspension was then diluted with water so that each 6-inch pot of soil received 1/25, 1/50 or 1/150 of the contents of one Petri-dish in 20 ml of inoculum.

Plants, in pots not inoculated with the fungus, were harvested at 0, 2, 5 and 11 days after inoculation and extracts of hypocotyls prepared and analyzed for sugars and Nh-pos substances as previously described.

The plants from which the extractions were made were grown in one growth chamber with each temperature regime being set at a different time. Later, two small growth chambers became available and the disease tests were repeated so that the temperature regimes of 30 D, 20 N and 20 D, 10 N could be tested concurrently. In this way, any differences in inoculum between tests were eliminated. Inoculations in the small growth chambers were made on the hypocotyls of emerged seedlings by the method previously described. The inoculum was allowed to run on to the hypocotyl at the soil surface.

A portion of each of the extracts mentioned above was also used as a medium to measure the amount of fungal growth that could be supported. To prepare the media, the plant extracts were thawed, centrifuged and then sterilized by passage through sintered glass filters. Before sterilization, the volume of each extract was adjusted with deionized water so that equal volumes of extract were derived from equal dry weights of hypocotyls.

Seventy-five ml of sterile filtrate were added to each sterilized 125-ml erlenmeyer flask which was plugged with cotton. One 7-mm disc cut from a 1-week-old, Petri-dish culture of the fungus on PDA was used to seed each flask of sterile plant extract. After 6 days growth at 25°C, the mycelial mats were separated from the liquid media by filtering on a Buchner funnel. The mats were then dried in an oven overnight at 80°C and weighed after cooling in a desiccator. At the time of

seeding the flasks, several 7-mm discs like those used for the inoculum were dried and weighed. The mean weight of these discs was subtracted from the weights of the mycelial mats after 6 days growth. Preliminary cultural tests to determine the effect of pH on growth of the fungus, the amount of medium to use, and the harvest date for maximum growth are reported in Appendix B.

Results

Total sugar concentrations, in general, increased during the 11-day growth period at all temperature regimes; however, the greatest increase was at the lowest temperature regime (Figure 2A).

The concentration of Nh-pos substances decreased with time and the greatest decrease was at the highest temperature regime (Figure 2B).

Growth of R. solani was somewhat variable but was greatest in extracts from plants grown at the lowest temperature regime and least in extracts from plants grown at the highest temperature regime (Figure 2C).

The survival of seedlings grown in R. solani infested soil was lowest at the highest temperature regime (30 D, 20 N), somewhat higher at the lowest temperature regime (20 D, 10 N), and still higher at the 25 D, 15 N regime (Table III).

When plants were inoculated on the hypocotyl, the mean disease indices were as follows:

| Temperature regime | Mean disease index |
|---|---|
| 30 D, 20 N | 3.3 |
| 20 D, 10 N | 2.8 |

Thus both disease tests in pots of soil indicated that the disease was greater at the 30 D, 20 N regime than at the 20 D, 10 N regime.

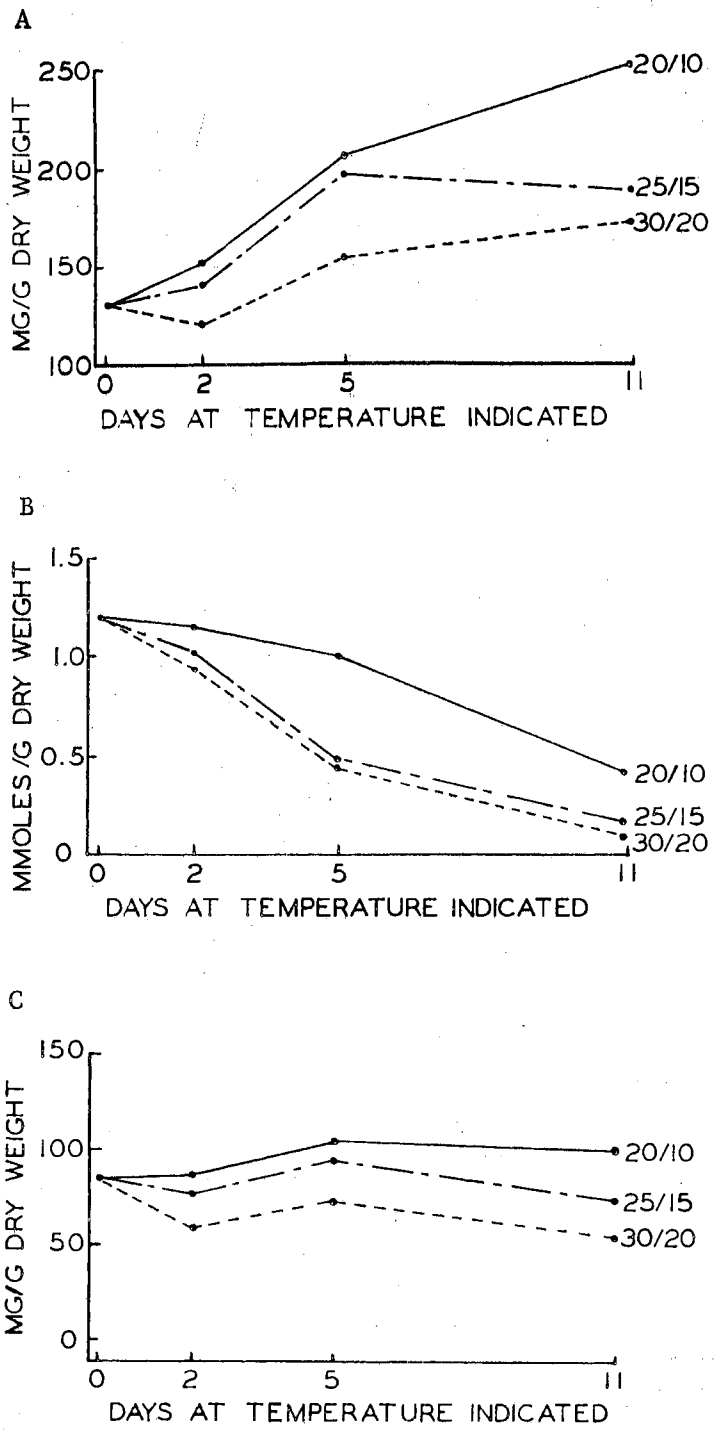


Figure 2. Effect of Temperature and Age on A) Total Sugars, B) Nh-pos substances, and C) Growth of R. solàni in Extracts of Hypocotyls.

The results showed that the concentration of sugars in hypocotyls was related to growth of the fungus *in vitro* but both of these factors were inversely related to the amount of disease under the conditions of this series of experiments.

## CHAPTER III

## TEMPERATURE RELATIONSHIPS

Results obtained with plants grown in nutrient solutions in growth chambers indicated that increased sugars might contribute to increased disease levels at lower temperatures. This view was strengthened by increased *in vitro* growth of *R. solani* in extracts obtained from plants grown in pots of soil in growth chambers at low compared to higher temperatures. However, in the soil tests, the incidence of the disease was found to be less at the lower temperatures. It was apparent that the concentration of sugars present in the hypocotyl was not the only factor affecting disease expression. A possible explanation was sought in the temperature relationships existing in pots of soil and containers of nutrient solutions.

Temperatures were measured with thermistors at 1 and 4 inch levels in 6 inch pots of soil, and between the polyurethane strips and at a depth of 4 inches in the containers of nutrient solution at a temperature regime of 30°C day and 15°C night. These temperatures were compared to air temperatures in the chambers recorded at the same time.

The effect of temperature on growth of the *R. solani* isolate was also studied at this time in Petri plates of peptone-dextrose-agar. A 7-mm disc from a 3-day old culture of the fungus was placed in the center of each plate. Six plates were placed in each of 6 incubators maintained at the following temperatures: 10, 18, 25, 30, 35 and 40°C.

The diameters of the colonies were measured every 24 hours. After 48 hours, 3 of the 6 plates from the 10 and 40° C incubators were placed at 30°C.

From this study it was found that the optimum temperature for growth of the R. solani isolate was approximately 30°C (Figure 3). Thus, the higher temperature regimes used in the plant growth chambers would be more favorable for growth of the fungus than the lower temperature regimes. Visual observations on the size of plants grown at the different temperatures revealed that plant growth was also favored by the higher temperature regimes.

Changes in temperatures measured at both the 1 and 4-inch depths in pots of soil in the growth chambers did not lag much behind changes in air temperatures because of the relatively small volume of soil (Figure 4). Changes in temperature recorded at the 4-inch depth in nutrient solutions lagged behind changes in air temperature, whereas changes in temperature in the polyurethane foam, where the fungus was placed, more closely followed changes in air temperature. Temperatures measured by glass thermometers at intervals during the daylight period at other temperature regimes showed that the same temperature relationships probably hold for all the temperature regimes used in these studies.

Thus when root temperatures in pots of soil were unfavorable for growth of plants in the 20 D, 10 N regime, temperatures near the soil surface were also low and unfavorable for growth of the fungus. Higher temperatures in the 30 D, 10 N regime were favorable for both the fungus and the plant. It is postulated that of the temperature regimes used with pots of soil, the 20 D, 10 N regime was less favorable for

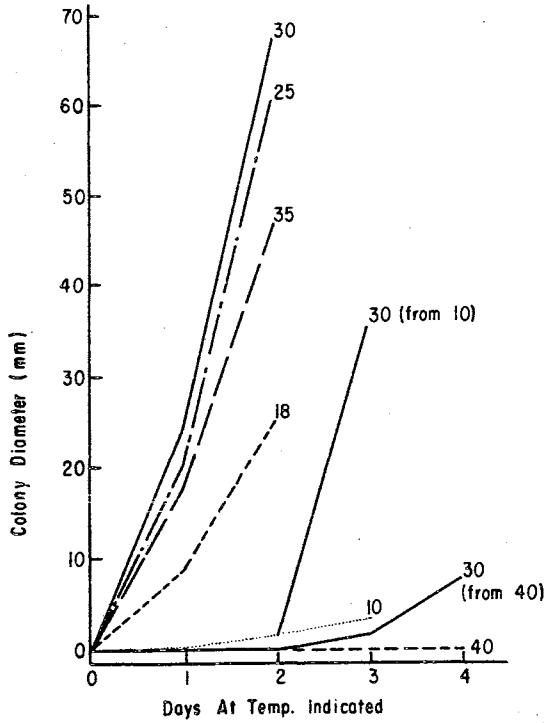


Figure 3. Effect of Temperature on Radial Growth of R. solani. Half of the Petri Plates Were Moved From 10, and 40 to 30°C After 2 Days.

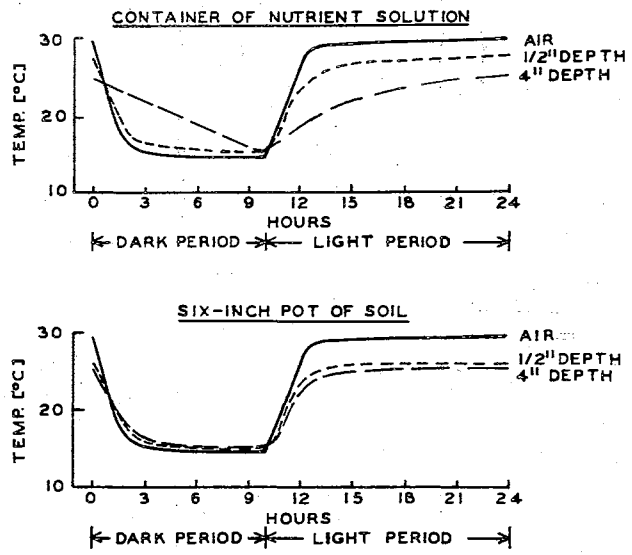


Figure 4. Temperatures Recorded in a Growth Chamber.

the fungus than for the plant, while the 30 D, 20 N regime was more favorable for the fungus than for the plant.

In containers of nutrient solution the roots of the plant and the fungus were seldom at the same temperature. During the day the fungus was at a higher temperature than the roots of the plant. Thus a temperature relationship existed whereby at a low temperature regime, low root temperatures were unfavorable for plant growth, but higher temperatures in the polyurethane foam enabled the fungus to grow. At a higher temperature regime, the difference in temperatures in the root zone and in the zone of inoculum was less advantageous to the fungus.

The temperature relationship obtained in containers of nutrient solution rather than in pots of soil more closely resembled temperature relations in the field. Means of temperatures recorded during two cotton planting seasons in Oklahoma were 1.5 to 3°C higher at the 1 inch than at the 6 inch depth.

The incidence of disease in buckets of nutrient solution rather than in pots of soil also more closely approached observations on disease development in the field where the disease is more severe at lower temperatures.

# CHAPTER IV

## TEMPERATURE STUDIES IN THE GREENHOUSE

To study in more detail the relation of temperature to certain biochemical parameters of the plant and seedling disease expression, the effect of four root temperatures on the following was investigated: (i) Concentrations of reducing sugars, total sugars, starch, nitrogen, and Nh-pos substances in hypocotyls; (ii) Growth of R. solani in ethanolic extracts of dried hypocotyls; and (iii) Disease severity in inoculated plants.

### Methods and Materials

Seedlings of cotton, cv CP 5 S1, were started and transferred to containers of nutrient solution as previously described. Sixteen containers were placed in each of 4 tanks of water, where the temperature was maintained at 10, 15, 20 or 30 $\pm$ 0.8°C. Air temperature ranged between 19 and 30°C and was not an experimental variable.

Hypocotyls were inoculated as previously described. Three different inoculation tests were made. In test 1, the hypocotyls were inoculated with the fungus suspension using one Petri plate of fungus per 300 ml water. In test 2, the inoculum consisted of 1 Petri plate of fungus per 100 ml, or 1 plate per 300 ml of water. Test 3 was the same as test 1. All inoculations were made on the 1st day following transplanting.

Non-inoculated plants from test 1 were harvested, as previously described, for chemical analyses at 0, 5, and 11 days after being placed in the temperature tanks. When the harvest was complete, the hypocotyls were cut into sections about 1.5 cm long, freeze-dried and then stored in a desiccator. For chemical analyses, the samples were ground to pass a 60-mesh screen and stored at room temperature in a desiccator. Extracts from 280-mg of the ground samples were made with three successive 30-ml portions of 80% ethanol at 80°C for 10 minutes each. The extracts were separated from the plant material by centrifugation and pooled. The ethanol was removed *in vacuo* (up to 80°C) with a rotary evaporator.

After the volume had been reduced to less than 30 ml, the remaining solution was quantitatively made up to 56 ml. Thirty-six ml of extract were diluted to 100 ml and frozen for later use as a medium for *R. solani*. Two ml of chloroform were added to the remaining 20 ml of extract, the mixture shaken vigorously, and then centrifuged. Ten ml of the upper phase were transferred to a test tube and frozen for subsequent determinations of sugars, Nh-pos substances, and nitrogen.

Nh-pos substances, reducing sugars and total sugars were determined as previously described.

Starch was extracted from the residue left from the ethanol extractions according to the method of Hassid and Neufeld (21). The amount of starch present in the resulting solution was determined by the method described for total sugars. Total nitrogen and ethanol-extractable nitrogen were determined by a modification of the Kjeldahl method as described by Humphries (23) using Hengar selenized granules as the catalyst in the digestion, and 0.01 N $H_2SO_4$ to titrate the

distillate-boric acid solution.

Plant extract media for growth of R. solani were prepared as previously described.

Two cultural tests employing a synthetic medium (see Appendix B) were made with the same isolate used in the above studies. In one test the concentrations of the nitrogen and carbon sources were varied so that 10, 30, 60, and 90 μmoles of asparagine, and 10, 20, and 40 mg of dextrose were in all possible combinations in each 80 ml of media. In the second test, different sources of carbon (citric acid, succinic acid, malic acid, fructose and sucrose) were used singly and in combination with glucose.

The basal synthetic medium was made up without the carbon source, the pH adjusted to 6.0, and 70 ml of the medium distributed to each 125-ml cotton-stoppered flask. The flasks of medium were then autoclaved at 120°C (15 psi) for 20 minutes. Solutions of the carbon sources were prepared separately, adjusted to a pH of 6.0 and filter-sterilized. Five or ten-ml portions of each carbon source were then added to the proper flasks. In the test with various amounts of asparagine and dextrose, the synthetic medium was prepared at 1/40 the original concentration. In the test of various carbon sources, the medium was 1/20 the original concentration with the carbon sources supplying 24 mg of carbon per flask.

The flasks were seeded with the fungus and harvested after 6 days as already described for the plant extract medium.

## Results

The concentration of total sugars increased at the two lower root

temperatures and decreased at the two higher root temperatures with time (Figure 5A). The concentration of reducing sugars did not significantly change at the 3 lower root temperatures, but did decrease at 30°C for the first 5 days, and then remained constant (Figure 5B). The concentration of starch increased at the two lower temperatures but did not change at the two higher temperatures (Figure 5C). The starch content was very low compared to the content of total sugars (less than 5%).

The concentration of Nh-pos substances decreased at all temperatures for 5 days, then remained unchanged at the 10°C root temperature, but continued to decrease at the other root temperatures (Figure 5D). The concentration of ethanol-extractable nitrogen followed the same pattern as, and was approximately equal to, the concentration of Nh-pos substances; consequently this data is not shown. The concentration of total nitrogen in the tissues decreased with each harvest period but was not directly correlated with root temperatures (Figure 5E).

The growth of R. solani was greatest in the plant extracts from the 10°C root temperature treatment and progressively less at higher temperature treatments (Figure 5F).

Disease development was greatest at the lowest root temperature and was progressively less as the root temperature increased (Table IV). This trend was true at both inoculum levels.

Growth of the fungus in synthetic media increased in response to an increase in either glucose or asparagine when the other was held constant (Figure 6A). The response to glucose was greater than to asparagine.

In the test of various carbon sources, glucose, fructose and su-

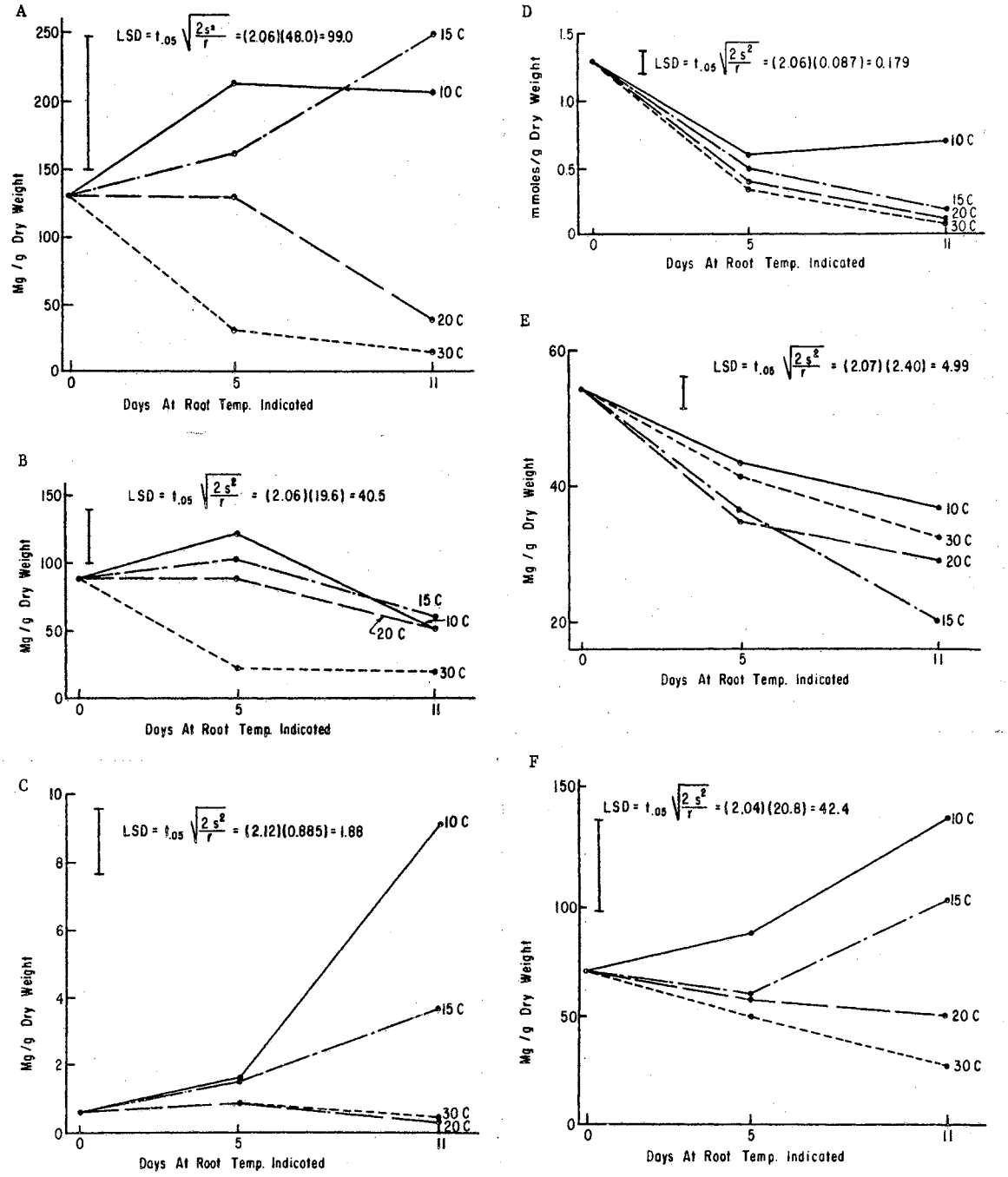


Figure 5. Effect of Root Temperature and Age on A) Total Sugars, B) Reducing Sugars, C) Starch, D) Nh-pos Substances and E) Nitrogen in Hypocotyls, and F) Growth of R. solani in Extracts of Hypocotyls.

TABLE IV

MEAN DISEASE INDICES[1] OF COTTON SEEDLINGS GROWN AT DIFFERENT CONSTANT ROOT TEMPERATURES AND INOCULATED WITH *R. SOLANI*

| Test No. | Days before inoc. | Conc. of inoculum | Root temp. (C) | | | |
|---|---|---|---|---|---|---|
| | | | 10 | 15 | 20 | 30 |
| 1 | 1 | 1X | 1.8 | 1.5 | 1.0 | 0.8 |
| 2 | 1 | 1X | 2.5 | 2.2 | 1.5 | 1.0 |
| 2 | 1 | 3X | 3.6 | 3.1 | 3.1 | 2.4 |
| 3 | 1 | 1X | 1.7 | 1.6 | 1.5 | 1.0 |

[1]Each index is a mean of disease grades from 4 replicates of 20 plants each. The disease grades were based on the following degrees of infection: 0 - none, 1 - slight, 2 - moderate, 3 - severe, 4 - dead plant.

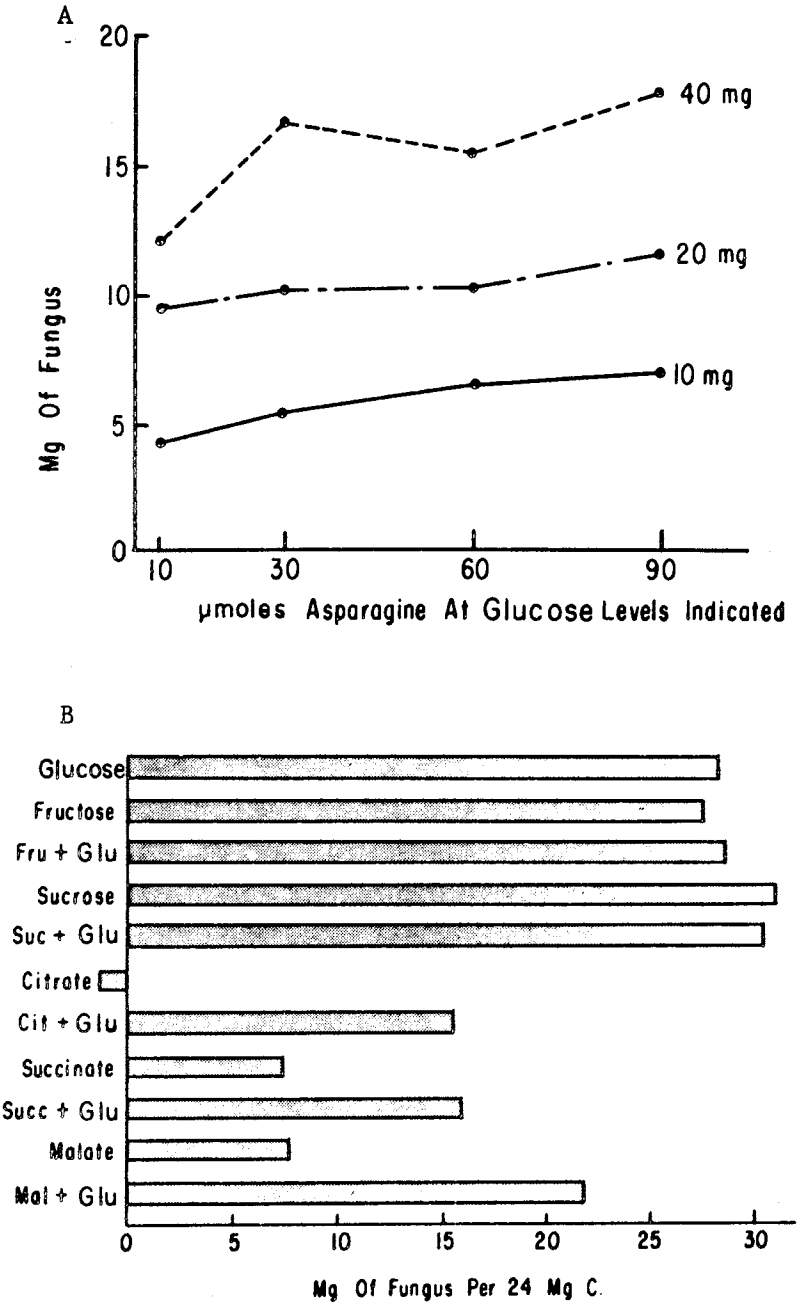


Figure 6. Growth of R. solani in Synthetic Media Containing Different A) Amounts of Asparagine and Glucose, and B) Carbon Sources.

crose and combinations of these sugars gave equally good growth (Figure 6B). Of the organic acids, citric acid gave no growth by itself and contributed nothing to growth when dextrose was present. Succinic and malic acids gave some growth when used alone. When used with glucose, malic apparently contributed to growth but succinic did not.

## Discussion

The data presented corroborates earlier evidence (20, and Chapters II and III) that low root temperatures result in increased total sugar concentration in the hypocotyls of cotton seedlings. With the same root temperatures and under the same conditions which resulted in an increase in concentration of total sugars, seedling disease due to R. solani increased. Hypocotyl extracts from plants at lower root temperatures supported more growth of the fungus in vitro than extracts from plants at higher root temperatures. Growth of the fungus in synthetic media increased in response to an increase in glucose. Thus, it appears that increases in sugars due to low root temperatures can be a contributing factor to increased seedling disease incited by R. solani. Sugars are a major source of carbon in the hypocotyls. Guinn and Hunter (20) found that the principal sugars present are sucrose, glucose, and fructose. Evidently any one or combinations of these are equally good carbon sources. The low starch content of the hypocotyls probably contributes little to the nutrition of the fungus.

Total nitrogen in hypocotyls seems to bear no relationship to disease severity as influenced by the effect of temperature on the plant. Nh-pos substances, however, decreased with each increase in root temperature. Thus, lower concentrations of Nh-pos substances are

associated with decreased susceptibility. However, as a carbon source their role in the nutrition of the fungus is certainly overshadowed by differences in the amount of sugars present. Their role as a source of nitrogen and their function in protein synthesis, as related to disease development under various conditions, should be studied further.

In addition to sugars and Nh-pos substances, organic acids may play a role in the nutrition of the fungus. Two of the three organic acids tested supported some growth of the fungus. The quantity of these acids present in cotton seedlings is not known.

# CHAPTER V

## AGE OF PLANTS

R. solani typically causes a disease of seedlings. The present chapter deals with the relationship of the content of sugars and Nh pos substances in hypocotyls to age of seedlings at the time of inoculation and to disease expression.

Plants were grown in pots of soil in growth chambers as described in Chapter II except that seeds were planted on different days so that seedlings, which had been at a regime of 25°C day, 15°C night for 0, 2, 5, and 11 days, were available for inoculation. Inoculations were made by placing 0.1 ml of the fungal suspension on each hypocotyl at the soil level. Plants were graded after 8 days.

Disease expression was greatest on the youngest plants and progressively less on the older plants (Table V).

The growth of R. solani in hypocotyl extracts was compared to the concentration of sugars and Nh pos substances present at each harvest period (Figure 7). This data was taken from Figures 2A, B and C. At this temperature regime, total sugars increased for 5 days and then remained constant; Nh-pos substances decreased with time. Growth of R. solani in hypocotyl extracts remained relatively constant and, apparently, was not related to the concentration of sugars or Nh-pos substances. The decrease in disease with age of seedlings was not related to the concentration of sugars in the hypocotyls but did parallel

TABLE V

THE EFFECT OF AGE OF SEEDLINGS ON DISEASE INCITED BY *R. SOLANI*

| Number of days at 25 D, 10 N before inoculation | 0 | 2 | 5 | 11 |
|---|---|---|---|---|
| Age of seedling | 5 | 7 | 10 | 16 |
| Mean disease index | 3.0 | 2.8 | 1.1 | 0.7 |

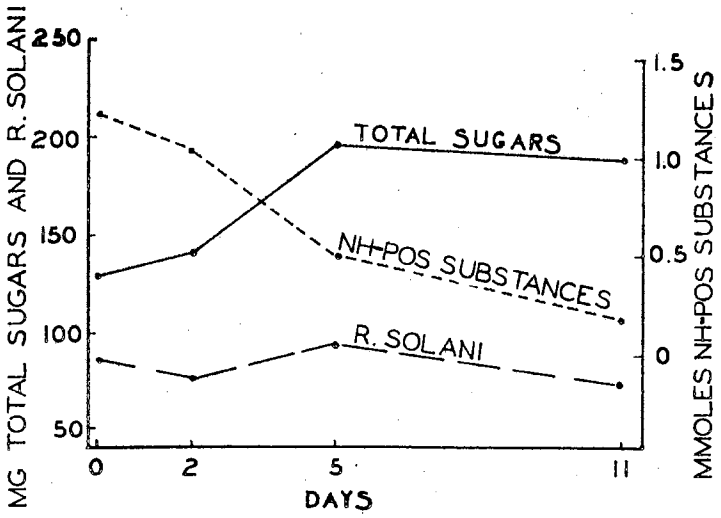


Figure 7. Effect of Age of Seedling on Total Sugars, Nh-pos Substances, and Growth of R. solani in Extracts of Hypocotyls. All Values are Given per Gram of Plant Dry Weight.

the decreasing concentration of Nh-pos substances. It appears that the decreasing susceptibility of cotton seedlings to disease incited by R. solani, which is associated with age, is not attributable to a decrease in sugar concentration in the hypocotyl. Some possible sources of resistance in older seedlings are secondary thickening and chemical changes in cell walls of the hypocotyl, a decrease in total or a change in amount of certain Nh-pos substances, the production of phytoalexins, and the presence of fungistatic substances. The last possibility was investigated and the findings presented in Chapter VII.

# CHAPTER VI

## NUTRIENT LEVELS

Nutrient solutions of four different concentrations were prepared and placed in a growth chamber at a temperature regime of 25°C day and 15°C night. The four nutrient solutions were prepared by using the salts containing N, P, K, Mg, and Ca at concentrations of 1, 0.1, 0.01 and 0 times that given in Table I. The other components of the nutrient solution were used at the 1X concentration. Uninoculated plants were harvested 7 days after being transfered to the nutrient solutions and treated as previously described (Chapter II) for chemical analyses and for extract media. Inoculations were made by placing 0.1 ml of a fungal suspension (1 plate/100 ml) on each hypocotyl between the polyurethane strips. Plants were graded for disease 8 days later.

The concentration of total sugars increased and that of Nh-pos substances decreased as the concentration of the nutrient solutions decreased (Figure 8). Growth of R. solani in the hypocotyl extracts increased as the nutrient level decreased except at the 0 nutrient concentration. The differences in fungus dry weights were small compared to the relatively large differences in total sugars. The decreasing concentrations of Nh-pos substances or of inorganic ions may have restricted growth of the fungus even though sufficient amounts of sugar were present.

The amount of seedling disease decreased with decreasing nutrient

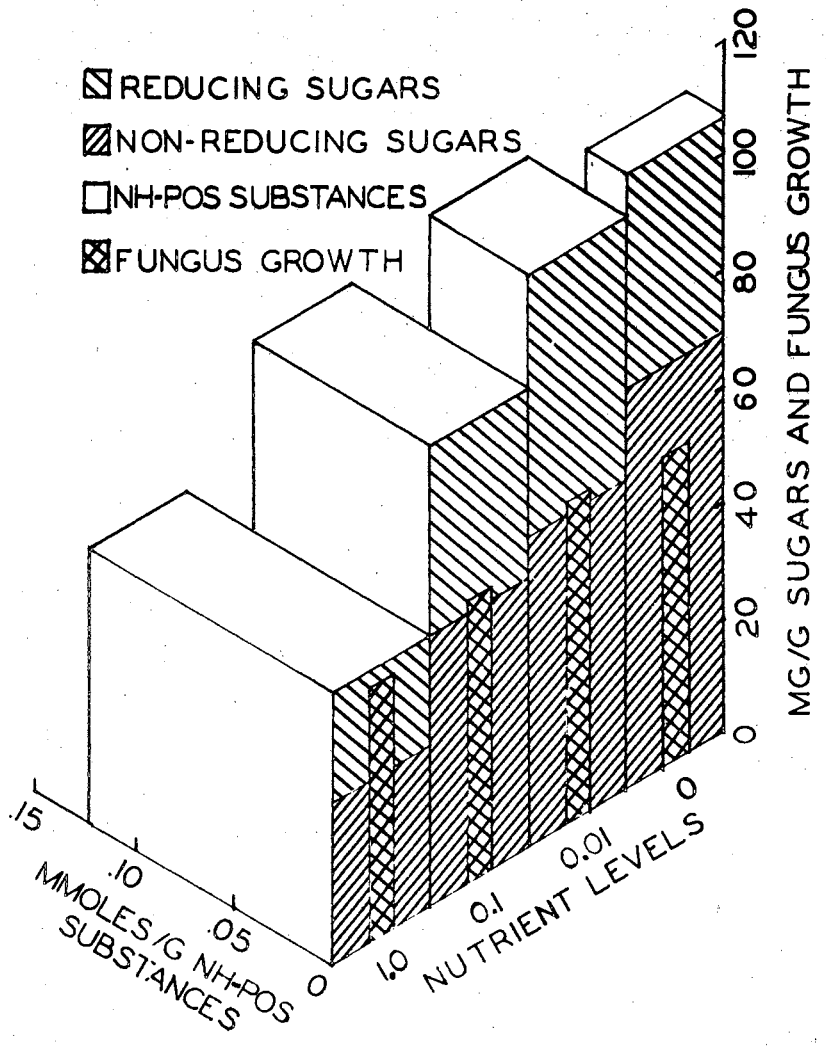


Figure 8. Effect of Nutrient Levels on the Chemical Composition of Hypocotyls and on Growth of R. solani in Extracts of Hypocotyls.

concentrations (Table VI) and was inversely related to total sugar concentrations.

The results from this experiment suggest that the concentration of total sugars present in hypocotyls was not a determining factor in pathogenesis. The fact that the total growth of the fungus in the extracts became proportionately less compared to the increasing total sugar concentrations, suggests that perhaps Nh-pos substances or inorganic ions became limiting factors to growth. The increase in growth of R. solani in the extracts (except at the 0 nutrient treatment) and the decrease in the disease index, when nutrient levels decreased, suggests additional possibilities. Perhaps the tissues of the plant became structurally more resistant, or a more plausible explanation is that the supply of inorganic ions in the nutrient solution became a limiting factor to growth of the fungus before pathogenesis.

TABLE VI

EFFECT OF NUTRIENT CONCENTRATIONS ON SEEDLING DISEASE INCITED BY R. SOLANI IN A GROWTH CHAMBER SET AT 25°C DAY, 15°C NIGHT

| Nutrient concentration[1] | Disease index |
|---|---|
| 1.0 | 1.5 |
| 0.1 | 1.0 |
| 0.01 | 0.6 |
| 0 | 0.4 |

[1]Concentrations of only the salts containing N, P, K, Mg, and Ca were changed.

# CHAPTER VII

## FUNGISTASIS IN PLANT TISSUES

During pathogenesis, the cotton seedling provides the nutrients necessary for growth of R. solani. However, in addition to the presence in the plant of nutrient substances there might also be toxic substances present which would inhibit growth of the fungus. These substances, if present, could be formed prior to or following infection. The possibility of inhibitory substances being present before infection was explored through the use of paper chromatography and bioassay of eluates from the chromatograms.

### Methods and Materials

Plants were grown in the greenhouse at four root temperatures and harvested as previously described (Chapter IV). Lyophilized, ground hypocotyl tissues were extracted by mixing 600 mg of tissue with 10 ml of water and 10 ml of ether at room temperature. The mixture was shaken every 5 minutes and after 20 minutes was centrifuged at 27,000 x g for 15 minutes. The ether was pipetted off and another 10 ml of water and 10 ml of ether added. The shaking and centrifuging was repeated and the two ether portions pooled.

Both the ether and water fractions were evaporated to dryness, redissolved in 1.5 ml of ether or water and then each fraction was streaked on Whatman #1 chromatography paper. Each 1.5 ml of extract

was applied as 6-inch streaks on 6 papers. The 6 papers of any one group were chromatographed at one time in a descending manner in a solvent of isopropanol-water-ammonium hydroxide (sp gr 0.88) (80:19:1) until the solvent front was 15 inches from the line of origin (about 17 hours). When dry, the papers were divided into ten equal strips parallel to the line of origin. An eleventh strip, which served as check, was cut from the paper in an area over which the solvent had run but which was at right angles to, and away from, the line of origin. Four of the papers were cut into strips which were then extracted with 210 ml of a synthetic culture medium. For these extractions, each four strips having the same $R_f$ value were cut into pieces about 4 cm square, soaked 5 minutes in the medium and then stirred for 10 minutes with a magnetic stirrer. The culture medium was then separated from the filter paper by filtering through glass wool. The medium was adjusted to a pH of 6 with 0.1 N $H_2SO_4$, and sterilized by passage through a sintered-glass filter. Sixty-ml portions of sterile medium were poured into sterile, cotton-stoppered, 125-ml erlenmeyer flasks. Three flasks were prepared from each of the 11 sectors. The flasks were seeded with R. solani and growth of the fungus measured as previously described.

The two chromatograms not used above were cut into strips about 2 inches wide at right angles to the line of origin. Each of these strips were treated as follows:

1. Sprayed with ninhydrin, 0.25% in n-butanol; heated at 80°C after spraying until zones appeared.

2. Sprayed with anisidine (1.2 g) and phthalic acid (1.7g per 100 ml 95% ethanol); heated at 120°C for 4 minutes after spraying.

3. Sprayed with sodium periodate (0.5% in water); then sprayed

with benzidine (0.5% in ethanol-acetic acid (4:1)) 5 minutes later.

4. Sprayed with bromcresol green (400 mg per liter of 95% ethanol).

One of the strips, before spraying, was placed under UV light and the fluorescent zones noted.

Chromatograms of pure substances were also run in the same solvent system and sprayed with the same reagents as the chromatograms of plant extracts. The substances used were glucose, fructose, sucrose, citric acid, fumaric acid, succinic acid, aspartic acid, asparagine, cystine, glutamic acid, lysine, glycine, serine, methionine, valine, and phenylalanine.

## Results

No differences were found in growth of the fungus in eluates from zones of chromatograms of the ether fractions. However, zones which stimulated and zones which inhibited growth of R. solani were found where the water phase was chromatographed (Figure 9, 10, 11, and 12). The amount of R. solani which grew in eluates from $R_f$ 0-.1 was greater than that of the controls in chromatograms from plants harvested at 0 days and at 11 days from root-temperature treatments of 10, 15 and 20° C. Growth in eluates from $R_f$ .2-.5 tended to be less than the controls and the difference was significant in at least parts of this zone in all the chromatograms except the one from plants harvested at 4 days from the root-temperature treatment of 10°C. The amount of inhibition of growth appeared to be greater in the extracts from 11-day hypocotyls compared to 4-day hypocotyls and also greater in extracts from hypocotyls grown at root temperatures of 20 and 30°C compared to 10

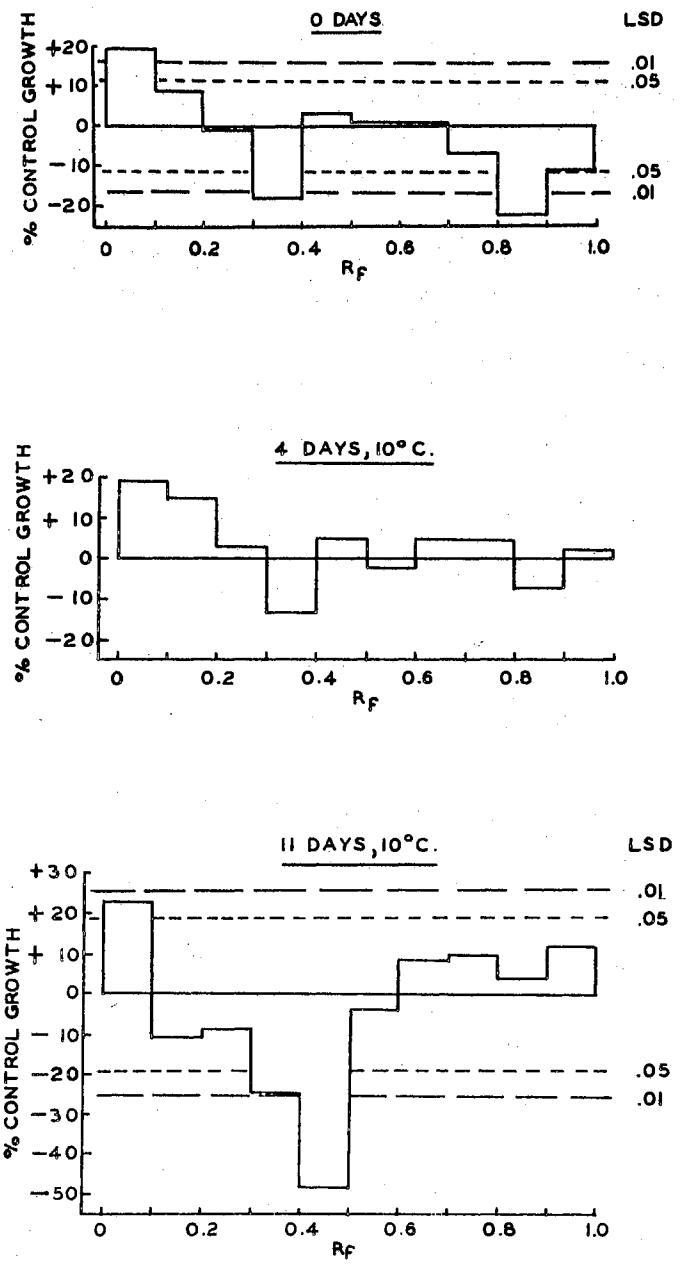


Figure 9. Bioassay of Chromatograms From Plants Grown for 0, 4 and 11 Days at a Root temperature of 10°C.

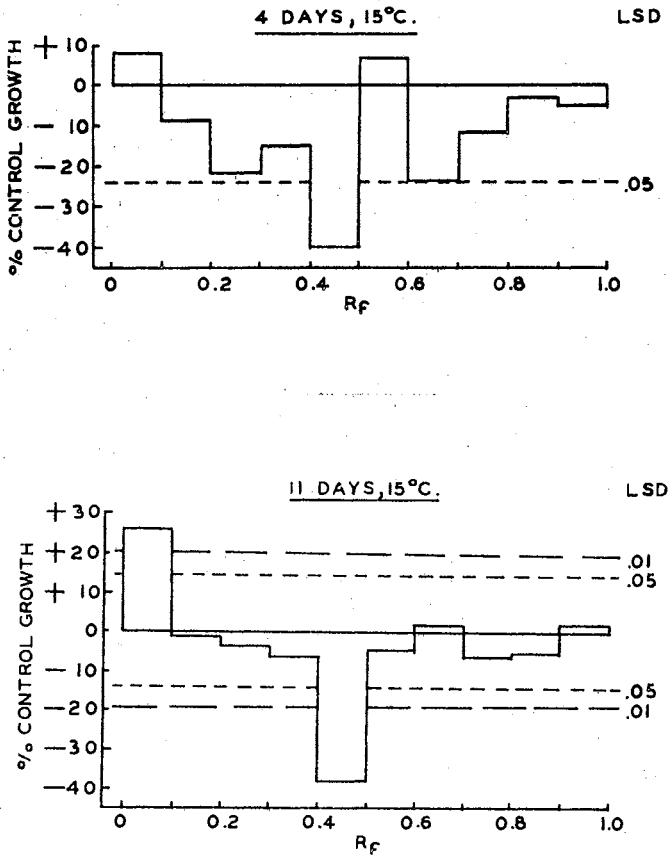


Figure 10. Bioassay of Chromatograms From Plants Grown for 4 and 11 Days at a Root Temperature of 15°C.

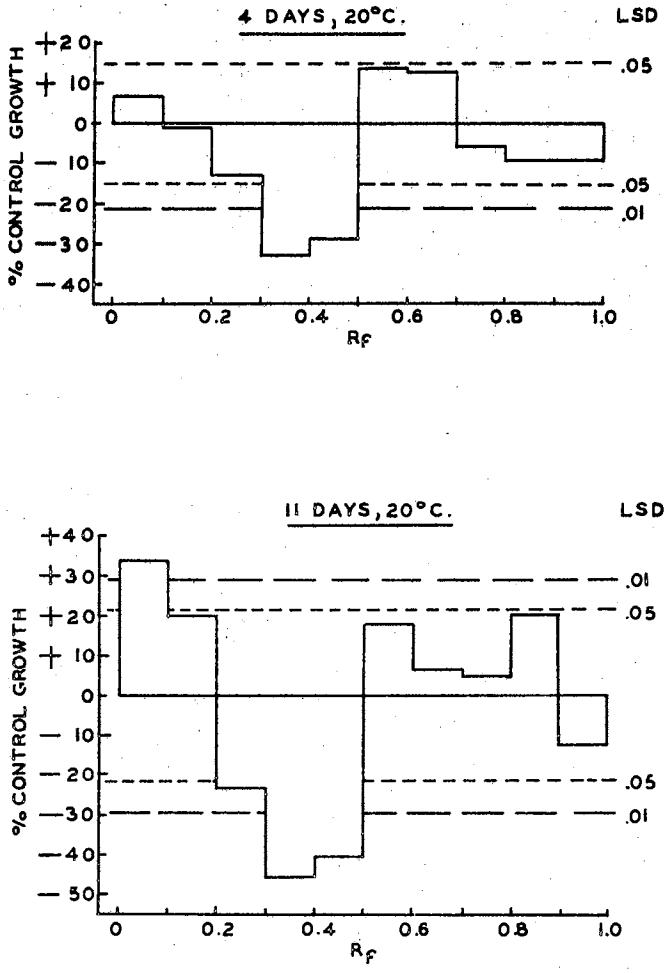


Figure 11. Bioassay of Chromatograms From Plants Grown for 4 and 11 Days at a Root Temperature of 20°C.

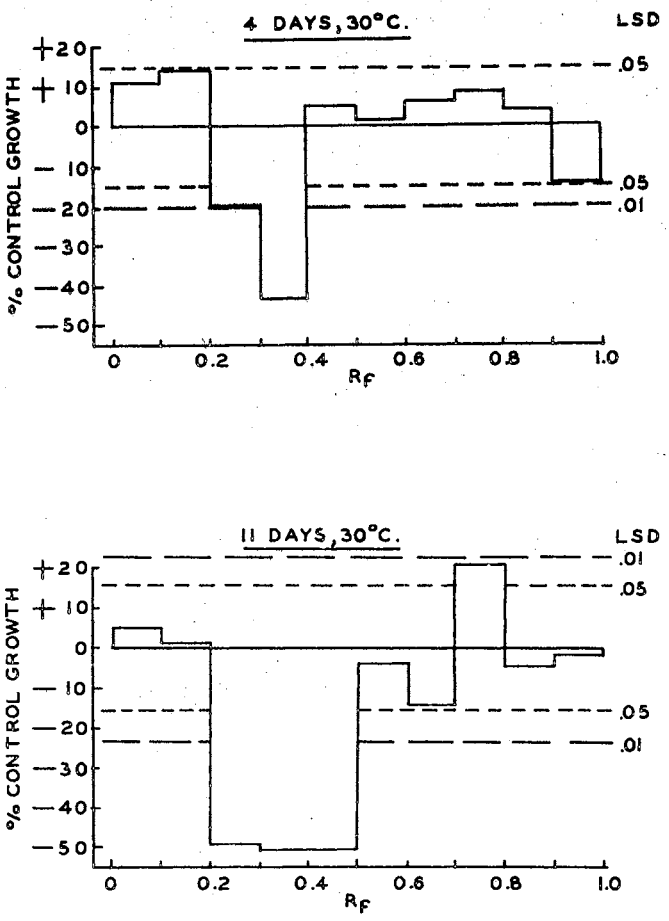


Figure 12. Bioassay of Chromatograms From Plants Grown for 4 and 11 Days at a Root Temperature of 30°C.

and 15°C.

The increased growth of the fungus in eluates from $R_f$ 0-.1 was not due to any sugars which could be identified. When known sugars were chromatographed, sucrose, fructose and glucose were located beyond $R_f$ .1 (Figure 13) and the oligosaccharides raffinose, melibiose, lactose, cellobiose and maltose were located between $R_f$ .1 and .2. The chromatograms of the extracts did contain materials in $R_f$ 0-.1, which gave a positive reaction to tests for reducing substances and polyols (Figure 13). Also in this zone were Nh-pos substances (Figure 14). No further attempt was made to identify the substance in $R_f$ 0-.1 which caused increased growth of R. solani.

The decreased growth of R. solani in eluates from $R_f$ .2-.5 could not be attributed to any particular type compound because none of the substances detected consistently fell within the zone of inhibited growth. One possible exception was in the acid zone, where at least part of an acid zone (Figure 15) always coincided with part of an inhibitory zone. It is worth noting that the leading zone of reducing substances was also located in or near the inhibitory zone. Presumably, this zone of reducing substances contained glucose and fructose which would tend to increase growth of the fungus if the inhibitory substance were not present.

The existence of a substance in hypocotyl extracts which is inhibitory to the fungus R. solani appears to be well substantiated. Further studies on this substance will require methods which would separate it from other materials present in the extracts.

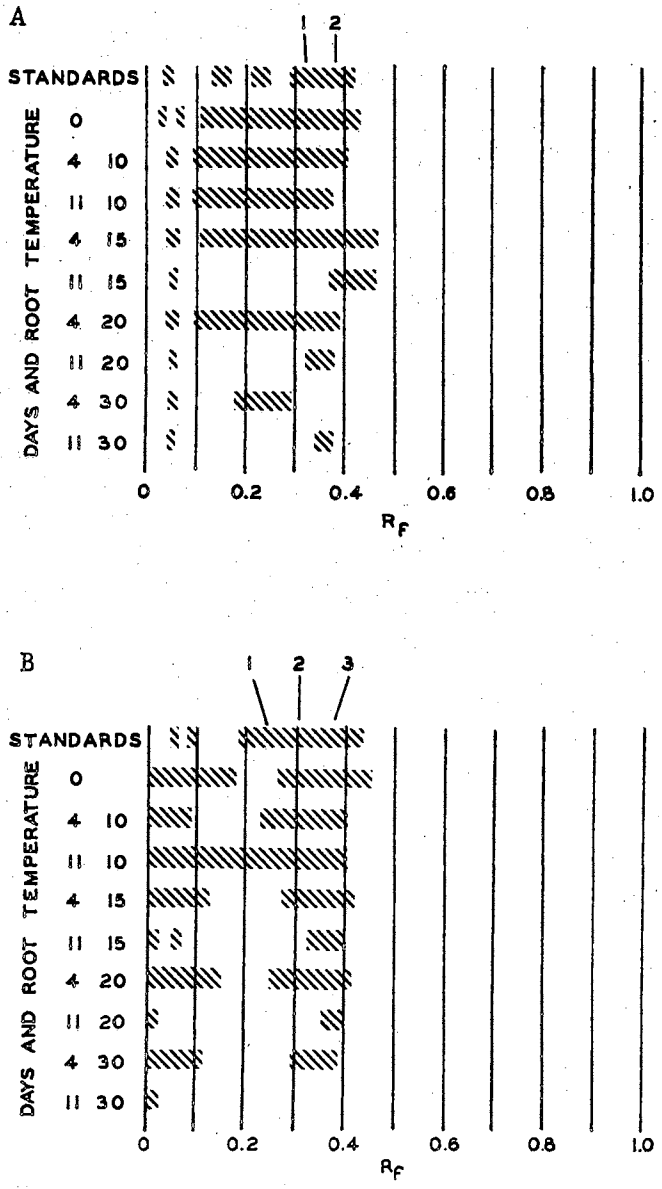


Figure 13. Location of A) Reducing Substances and B) Polyols on Chromatograms. Standards Used in A Were 1) Glucose, and 2) Fructose; Standards Used in B Were 1) Sucrose, 2) Glucose, and 3) Fructose.

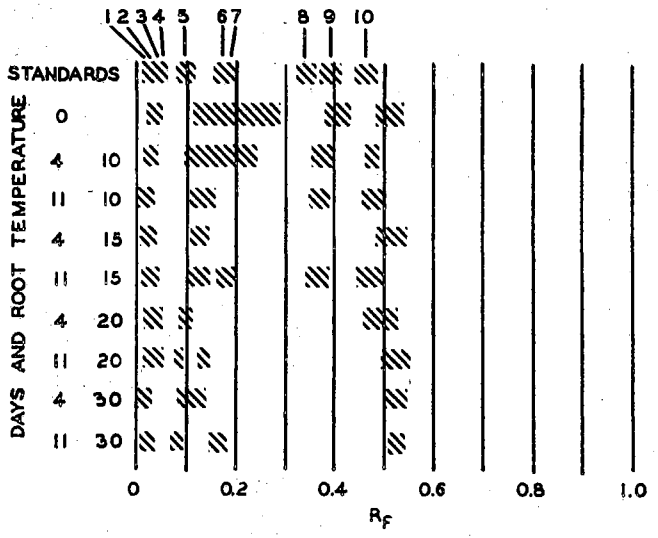


Figure 14. Location of Ninhydrin-positive Substances on Chromatograms, Standards Used Were 1) Aspartic Acid, 2) Asparagine, 3) Cystine, 4) Glutamic Acid, 5) Lysine, 6) Glycine, 7) Serine, 8) Methionine, 9) Valine, and 10) Phenylalanine.

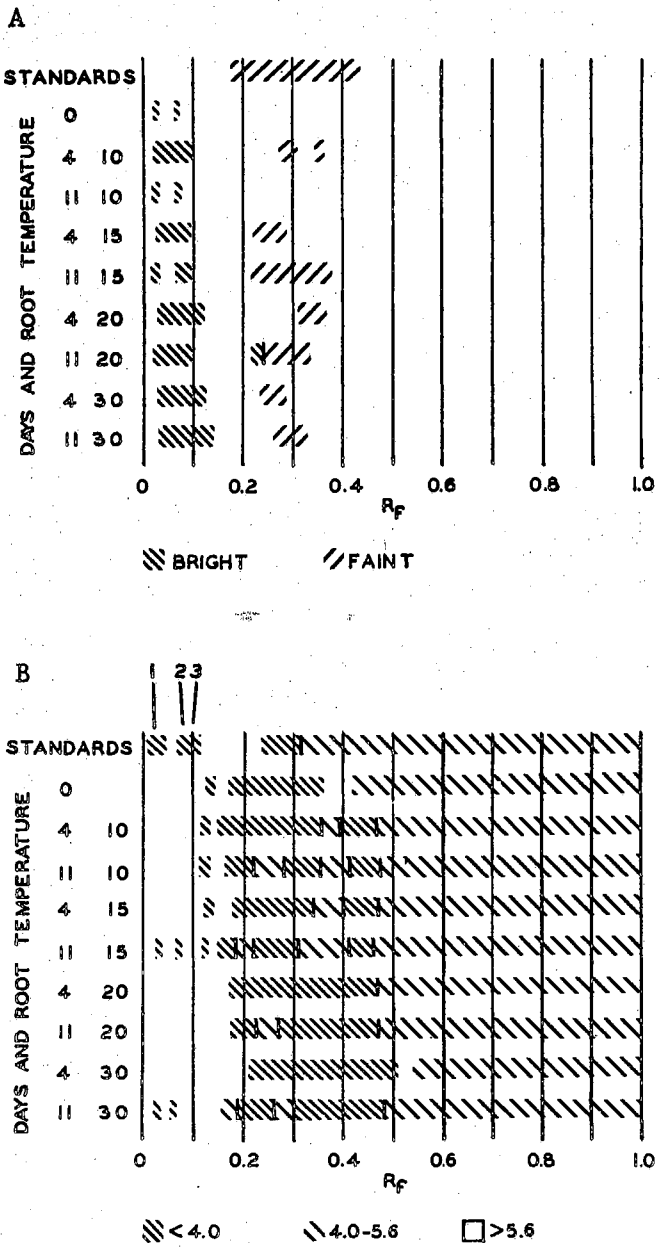


Figure 15. Location of A) Fluorescing and B) Acidic Substances on Chromatograms. Standards Used in B Were 1) Citric Acid, 2) Fumaric Acid, and 3) Succinic Acid.

# CHAPTER VIII

## DISCUSSION

Plants grown from seed go through a stage of growth during which they may be readily attacked by a group of organisms which incite seedling disease. The amount of damage incurred and the length of the period of susceptibility depend, to a great extent, on the environment. The effect of environment on the young rapidly growing seedling is especially noticeable; however, the pathogens involved are also influenced by environment. During pathogenesis the host and the pathogen are in intimate contact; therefore, environment affects the host or pathogen not only directly, but also indirectly. Most of the studies reported in this dissertation were concerned with the effect of temperature on certain biochemical compounds in the plant from the view point that the host is the source of nutrients for growth of the pathogen.

Weinhold and Bowman (47) found that R. solani must have certain quantities of sugars and amino acids available in order to function as a pathogen on cotton seedlings. It would be expected that a high level of nutrients would favor continued pathogenesis. In work reported elsewhere (20) and in this dissertation (Chapters II, IV) it was found that low root temperatures caused an accumulation of sugars in hypocotyls of cotton plants. The higher sugar levels were associated with increased seedling disease and favored increased growth of the fungus

*in vitro* (Chapter IV). In some cases, growth of the fungus in plant extracts was greater than could be accounted for by the amount of carbon supplied by the sugars and Nh-pos substances present. Other possible carbon sources for growth of *R. solani* are organic acids, and their occurrence in cotton seedlings is being studied further. In other instances, the amount of the fungus which grew in extracts did not increase in proportion to an increase in the amount of sugars present. If organic acids are a source of carbon, perhaps they did not increase as sugars increased. Other possibilities are that Nh-pos substances as a source of N became a limiting factor or inhibitory materials accumulated.

The level of total inorganic nutrients (N, P and K) may affect disease severity. Zyngas (49) reported that a change from low to medium levels of nutrients caused no change in disease severity but that a change from medium to high levels of nutrients caused a decrease in disease severity. In the present work (Chapter VI) increasing levels of nutrients were associated with increasing disease levels and with decreasing sugar concentrations in hypocotyls. The disease index was not related to growth of the fungus in extracts of hypocotyls. It is possible that in this case the structure of the plant tissues was an important factor affecting disease severity. Another possibility is that growth of the fungus as well as that of the plant was adversely affected by lower levels of inorganic ions. Zyngas (49) showed that low levels of N, P, K, Mg or S could limit growth of *R. solani*, *in vitro*. The results cited above in respect to levels of nutrients are not contradictory to Zyngas' work since the highest level of nutrients used in the present work was comparable to the lowest level of nu-

trients used by Zyngas. The effect of various levels of inorganic ions on disease severity deserves further study from the point of view of both the pathogen and the host. Temperatures further complicate this relationship since ion uptake by plants is at least partly dependent on water uptake which in turn is restricted by lower temperatures.

The influence of temperature on the seedling disease of cotton incited by R. solani has been extensively investigated and it has been found that low temperatures (2, 27, 39, 46) as well as high temperature (41, 45) increase disease severity while disease resulting from some isolates is not affected within a certain range of temperatures (31). While the conflicting results may be partly due to the fact that different isolates were used by the various workers, the method by which the temperature conditions were imposed is an important consideration. I found (Chapter II) that under one set of conditions the disease was more severe at the higher of two temperature regimes while under another set of conditions, but with the same isolate of R. solani, the disease was more severe at the lower of the same two temperature regimes. These different results obtained were attributed to the experimental methods which either favored the host or the pathogen at each temperature regime used (Chapter III). In these studies it was concluded that containers of nutrient solution rather than 6-inch pots of soil more closely resembled temperature conditions in the field, and also more closely resembled disease conditions in that seedling disease is commonly observed to be greater under cool rather than warm temperatures. The temperature relationships observed in the field and in buckets of nutrient solution, which favor increased seedling disease due to R. solani at lower temperatures, are low root temperatures which

slow growth of the host and cause an accumulation of sugars, and slightly higher temperatures in the hypocotyl region which allow the fungus to grow and attack the plant.

Another effect of temperature on expression of the disease may be on the production by the plant of substances inhibitory to growth of the fungus. Evidence was presented (Chapter VII) which suggested that fungistatic substances are present in cotton seedlings in greater quantities when grown at root temperatures of 20 and 30°C compared to 10 and 15°C. The identities of these substances have not yet been determined.

The fungistatic substances were found in healthy plants. Bell (9) found that gossypol was produced in cotton in response to inoculation with Verticillium albo-atrum and believes this substance is a phytoalexin. However, in young tissues (1-2 week old stems) only 10 to 20% as much gossypol was produced as in stems six weeks old. Pierre and Bateman (37) found that bean seedlings produce phytoalexins in response to infection with R. solani and concluded that this is an important factor contributing to host resistance in young bean hypocotyls. The role of phytoalexins on seedling diseases, especially in relation to environmental factors and host varieties, might prove to be a fruitful field of study.

Bateman and Lumsden (8) suggested that a decrease in ethanol-extractable substances (primarily sugars) found in older bean seedlings might be a contributing factor to the increasing resistance to R. solani associated with age of seedlings. I found that under a temperature regime at which cotton plants became more resistant with age, total sugars actually increased for the first five days but growth of

R. solani in extracts of hypocotyls was relatively constant (Chapter V). In effect, the extracts from older plants could have supported more growth of the fungus because of the higher sugar content, but they did not. The results from this and other experiments suggested the possibility of the existence of fungistatic substances. The decrease in amino acids may also be related to restricted growth of the fungus in the extracts and to the decrease in disease severity. Another contributing factor may be the formation of wound cork which Balls (5) suggested. He found that cotton seedlings were more resistant to the disease incited by R. solani when grown at 33 and 37°C than at 20 and 25°C. He attributed the increased resistance to the formation of callus tissue by the plant. Van Etten, Maxwell, and Bateman (44) found no evidence of wound periderm formation in the bean plant in response to infection with R. solani in the early stages of pathogenesis but beleive this may be important in older tissues.

Only a few of the numerous environmental factors contributing to susceptibility or resistance to this seedling disease have been discussed above. The possible mechanisms through which the environmental factors act in the host, pathogen, or both, are even more numerous. In addition to the number of possible mechanisms, a mechanism important to the disease syndrone at one stage of growth of the plant may be less important at a later stage of growth. The results from each experiment are like the pieces of a jigsaw puzzle. With each piece of experimental evidence which is fitted into place the total picture becomes more coherent; or perhaps evidence which formerly was out of place now becomes part of the whole. It is hoped that the present dissertation has contributed to a more coherent picture of the role that environment

plays in the seedling disease complex of cotton.

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# APPENDIX A

## METHOD OF SUPPORTING PLANTS IN NUTRIENT SOLUTION

The method of supporting plants in nutrient solutions was developed for work reported on in this dissertation. The method was described in Plant Disease Reporter 52: 148-159 and a reprint of this article follows on the next two pages.

# A METHOD OF SUPPORTING PLANTS IN NUTRIENT SOLUTION

R. E. Hunter

Growing plants in nutrient solutions has proved useful in a number of situations. A common method of supporting the plants is to place them in holes in the cover of the container of nutrient solution and then to fill the space between the plant stem and the edge of the hole with cotton. Such a method involves considerable time when large numbers of seedlings are used. This paper describes another method of supporting plants, which we have used in certain types of disease studies with cotton (Gossypium hirsutum) and have found it to have several advantages over other methods. A diagram of the apparatus is shown in Figure 1.

To transplant the seedlings to nutrient solutions, we germinate cottonseeds in vermiculite and remove them when the cotyledons have expanded. Up to 10 seedlings are placed along one of the polyurethane strips (Fig. 2), and another strip is placed over the seedlings. The resulting "sandwich" is placed in a slot in the wooden lid (Fig. 3).

For inoculations with Rhizoctonia solani, we insert the tip of a pipette between the polyurethane strips and allow a small amount of fungal suspension to run onto each hypocotyl. If desired, the strips can be removed from the lids at any time for observation of the disease; and then the strips can be put back without injury to the plants. To provide moisture around the hypocotyls so that the inoculum does not dry out, a 9-inch square of cheesecloth is placed over the polyurethane foam strip before the seedlings are laid in place. When the strips are in place in the wooden lid, the cheesecloth will hang into the solution and act as a wick.

The polyurethane strips will continue to support seedlings as they grow (Fig. 4). This method of supporting plants has also been used in cotton disease studies with Verticillium albo-atrum. We have made inoculations in these studies by simply pouring a suspension of conidia into the nutrient solution.

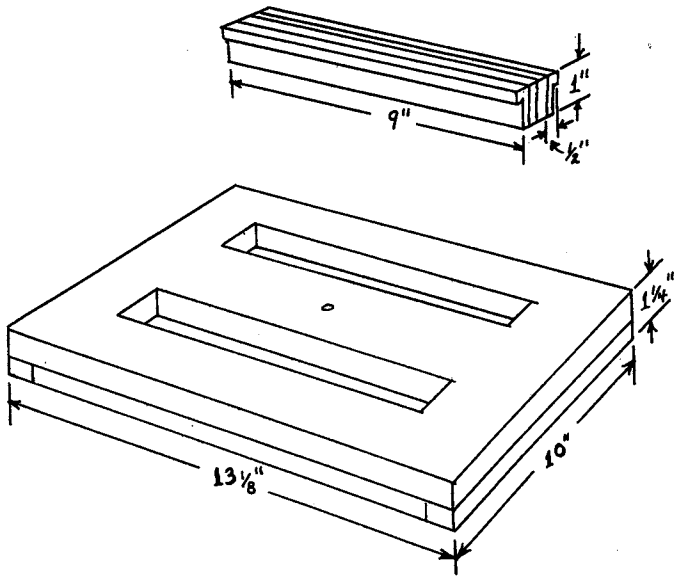


**Figure 16.** The Tops Are Made From 3/4-inch Plywood With 1/2 x 1-inch Strips of Wood Glued to the Bottom. Sheets of 1/2-inch Polyurethane Foam Are Cut Into 1 x 9 Inch Strips and Glued to the Redwood Strips. The Slots in the Plywood Tops Are Cut Slightly Narrower Than the Thickness of the Redwood-polyurethane "Sandwiches."

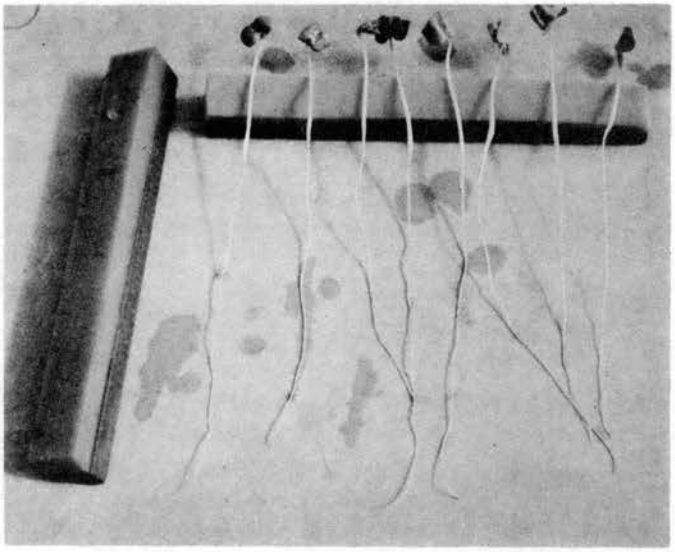

Figure 17. Cotton Seedlings in Place on the Polyurethane Foam. If a Cheesecloth Wick Is Used, Place It on the Foam Strip Before the Seedlings Are in Place.

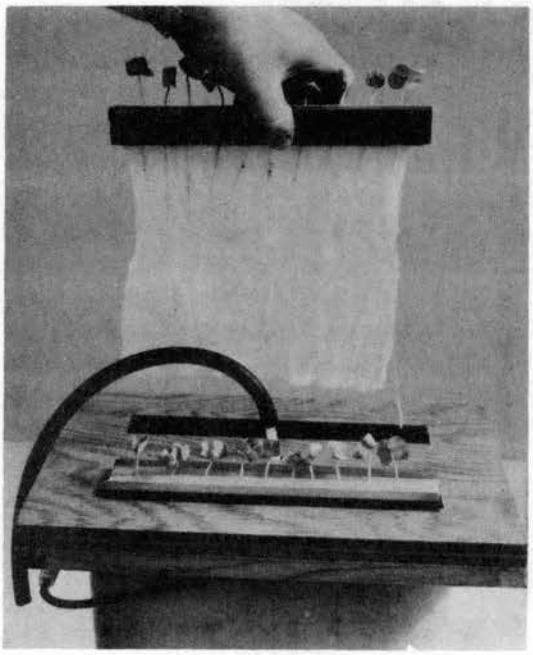

Figure 18. The Completed "Sandwich" With a Cheesecloth Wick, Ready to Be Placed in the Slot in the Plywood Top.

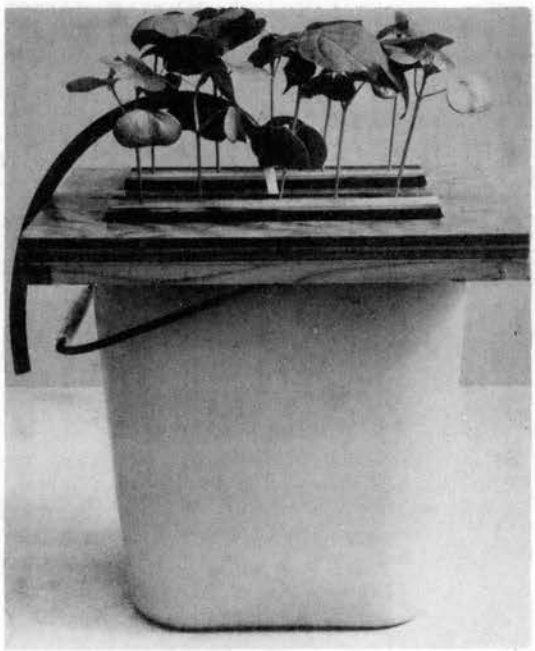

Figure 19. Cotton Seedlings About 2 Weeks Old Held Between Polyurethane Strips Over a Nutrient Solution. The Black Hose Is Connected to an Air Line for Aeration of the Solution.

## APPENDIX B

## FUNGUS CULTURAL STUDIES

Before the experiments involving growth of the fungus in plant extract or synthetic media were conducted, several preliminary studies were made to determine the following:

1. The age at which the fungus cultures should be harvested for maximum growth.

2. How much medium would be required in each flask to give an appreciable yield of fungus.

3. The pH at which growth of the fungus would be retarded.

Medium was prepared from freshly ground stems, filter sterilized, and 75 ml of medium was placed in each of 20 125-ml erlenmeyer flasks. Each flask was seeded with a 7-mm disk from a R. solani PDA culture and placed at 25°C. Four flasks were harvested at 2-day intervals and the dry weights of the fungus determined.

The maximum amount of growth of the fungus was obtained in this medium after 6 days (Table VII).

Forty-eight 125-ml erlenmeyer flasks of a basal synthetic medium (30) (Table VIII) diluted 1:40 were prepared so that one-third of the flasks contained 20, 40, or 80 ml of medium. The medium contained 500 mg of dextrose and 50 mg of asparagine per liter which was in the range of the total sugar and amino acid concentrations of some of the plant extract media. The synthetic medium was autoclaved at 15 psi for 20

TABLE VII

GROWTH OF R. SOLANI IN 75 ML OF PLANT EXTRACT MEDIUM[1]

| Harvest Day | Fungus Growth (mg) |
|---|---|
| 2 | -0.6 |
| 4 | 5.3 |
| 6 | 7.1 |
| 8 | 4.7 |
| 10 | 5.5 |

[1]The extract contained approximately 62.4 mg of total sugars and 66.5 mg of amino acids (based on the molecular weight of alanine) per liter.

TABLE VIII

BASAL SYNTHETIC MEDIUM

| Material | Amount |
|---|---|
| $KH_2PO_4$ | 1.0 g |
| $MgSO_4 \cdot 7H_2O$ | 0.5 g |
| Microelements[1] | 2 ml |
| Thiamine hydrochloride | 100 µg |
| Biotin | 5 µg |
| Water to make | 1000 ml |

[1]The microelement solution contained 723.5 mg of $Fe(NO_3)_3 \cdot 9H_2O$, 439.8 mg of $ZnSO_4 \cdot 7H_2O$ and 203.0 mg of $MnSO_4 \cdot 4H_2O$ dissolved in distilled water, sufficiently acidified with $H_2SO_4$ so that a clear solution resulted. The total volume was 1 liter.

minutes. The flasks were seeded as above, placed at 25°C and 12 flasks harvested after 4, 5, 6, and 7 days growth.

The amount of growth was meager in 20 ml of medium, appreciable in 40 ml and greatest in 80 ml with the maximum growth present after 6 days (Figure 20). From these two growth tests it was decided to use 80 ml of medium per flask, when available, and to harvest the fungus after 6 days.

The effect of pH on growth of the fungus was determined in a basal synthetic medium used at full strength and containing glucose (20g / liter) and asparagine (2g / liter) as the carbon and nitrogen sources. Four flasks of the medium (minus the carbohydrate) were adjusted to each of the following pH values: 3.5, 5.0, 6.5 and 8.0 before autoclaving. The glucose was prepared separately in a similar manner and then aseptically added to the proper flasks. Each 250 ml flask contained 30 ml of synthetic media. The flasks were seeded, placed at 25°C and harvested after 6 days at which time the pH of the medium was again measured.

Initial pH's of 5.0, 6.5, and 8.0 had little effect on growth of the fungus (Table IX). The final pH's in these 3 media were 4.1, 5.4, and 6.1, respectively. Growth was slightly less at an initial pH of 3.5 with a final pH of 3.3.

In all of the cultural tests conducted for this dissertation, the final pH of any medium was never less than 4.0. From the results of the above test it seemed reasonable to conclude that pH had little if any effect on growth of the fungus in these investigations.

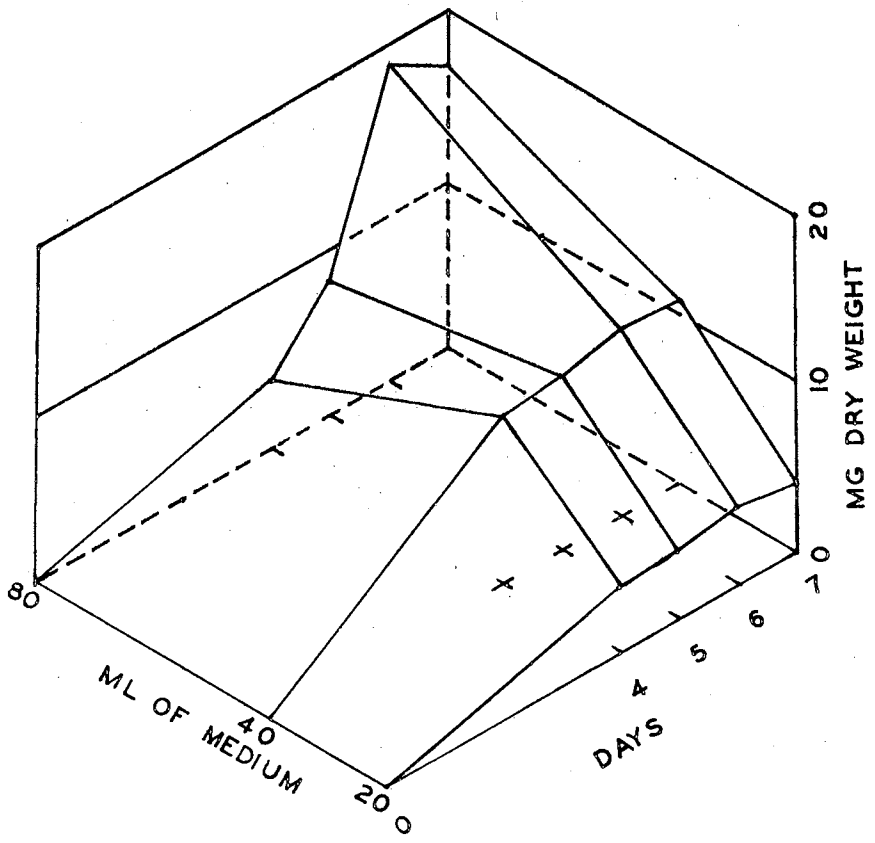


Figure 20. Growth of R. solani in Various Amounts of Medium and at Different Harvest Periods.

TABLE IX

EFFECT OF pH ON GROWTH OF R. SOLANI IN A SYNTHETIC MEDIUM

| Initial pH | Mg of fungus | Final pH |
|---|---|---|
| 3.5 | 347 | 3.3 |
| 5.0 | 359 | 4.1 |
| 6.5 | 358 | 5.4 |
| 8.0 | 363 | 6.1 |

VITA

Richard Edmund Hunter

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE EFFECT OF TEMPERATURE ON THE CHEMICAL COMPOSITION OF HYPOCOTYLS OF COTTON AND ON THE SEEDLING DISEASE INCITED BY RHIZOCTONIA SOLANI

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