# STUDIES ON THE PARASITISM OF STREPTOMYCES SCABIES (THAXT.) WAKSMAN AND HENRICI

A

Thesis

by

C. Harold Lawrence

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# INTRODUCTION

There is an increasing interest among Plant Pathologists to explore, more fully, the nature of parasitism. This interest lies in obtaining a clear understanding of the host-parasite relationship. The emphasis is placed on the physiological mechanisms involved which enables the plant pathogenic organisms to effect entry into the host body and to assimilate the available nutrients, to tolerate or overcome the resistance of the host and to induce disease in the host by their metabolic products or toxins.

The host-parasite relationship of the common scab disease of potatoes, caused by Streptomyces scabies (Thaxt.) Waksman and Henrici, presents many features of parasitism which would be interesting to explore as far as the physiological mechanisms are concerned. This disease is found in all parts of the world where potatoes are grown. It is primarily a potato tuber disease but has been reported on other crops such as beets, turnips and radish. It has been generally accepted that the disease attacks tubers at all stages of growth until fully ripened. My observations agree generally with those of previous workers in that the disease first appears as a small brownish spot or stain on the surface of the potato. The lenticels and stomata appear to be the chief avenues of infection. However, it has been shown by Lutman(1913) to originate on any portion of the potato.

Although early workers such as Millard and Burr (1926) recognized at least six types of scab lesions, the shallow and deep scab are two phases commonly referred to in popular and scientific literature. Shallow scab consists of a superficial roughened area, sometimes raised above, and often slightly below the plane of the healthy skin. It consists of corky tissue which arises from abnormal proliferation of the cells of the periderm of the tuber, resulting from invasion by the pathogen. The lesions vary widely in size and shape and usually are darker than the healthy skin. Scab spots once started, rapidly spread and increase in size with several infected spots often coalescing to form large patches or the whole exterior of the potato may become affected. The symptoms may be so mild that the tuber surface merely shows a diffuse russet appearance, or they may on the other hand, grade into the deep type of lesion.

Deep scab or pitted scab consists of lesions which are one to more than three millimeters deep. The pitted scab may originate in a tuber lenticel, the meristem of which is stimulated to divide giving rise to elongated cells. These cells are invaded by the parasites and subsequently collapse. As the destruction of the elongated cells proceeds the lenticel widens and after a time its meristem becomes less active; its last formed daughter cells cease to

elongate and become suberized, forming a compact tissue or barrier of wound cork cutting off the infected external cells from the tissue below. Further development is due to the organism growing through incompletely suberized cells that are present in the wound cork and infecting the cells below. Here another wound cork arises and if this barrier is incompletely suberized the organism may pass through it and again infect the cells below which enables a third wound cork to develop.

A great deal of work has been done on the control of this disease but as yet only a few general recommendations can be made. Considerable success can be obtained by keeping the soil, on which the potatoes are to be grown, highly acid (pH 5.2 to 5.4). This practice has the disadvantage in that crops that follow in the rotation usually require less acid soils for maximum production. The most effective way of controlling the disease is to plant scab-resistant potato varieties. There are a number of these available but as yet none are grown to any large extent. Most are only moderately resistant to the disease.

The ultimate aim of the Plant Pathologist is disease control and often this cannot be effected without a clear understanding of host-parasite relationships. This association in the common scab disease of potatoes is not

understood, consequently a study was initiated to determine some of the mechanisms involved in the parasitism of *S. scabies*.

## AIM AND SCOPE OF WORK

The primary aim of the work was to study some of the physiological mechanisms involved in the parasitism of Streptomyces scabies. The first of these studies was concerned with the effect of medium extracts from cultures of the organisms on the respiration of potato tuber tissue. The effect of extracts from three media were studied and determined by manometric techniques. A Warburg respirometer was utilized which was calibrated by standard procedures.

The second investigation was undertaken to determine whether certain parasitic strains of S. scabies as well as other Streptomyces species could produce some or all of the known pectolytic enzymes when these organisms were grown on potato tuber tissue media. The ultimate objective was to determine if any pectolytic enzymes produced would be an aid in separating pathogenic from nonpathogenic forms of the organism and aid in elucidating the host-parasite relationships.

In order to pursue these studies, cultures of known pathogenicity had to be selected. This necessitated a program of isolating the organisms from infested soils and scabby potatoes, and of testing their virulence and that of other available isolates as an indication of their suitability for the purposes of these studies.

# LITERATURE REVIEW

## RESPIRATION IN PLANT DISEASE

It is now generally recognized that the symptoms of a parasitic disease arise largely from an interchange of chemical substances between the invading parasite and its host. The concept of chemical substances as causative agents of disease implies the production by the parasite of diffusible metabolites having injurious effects upon the host. Whether these effects appear as morphological or physiological changes, they must ultimately be traced to changes in metabolism. Since respiration is the process which provides the source of available energy for life processes it affords an experimentally useful approach to the study of parasitism.

Respiratory changes in diseased tissues have been reported by many investigators. Furthermore, increased rates are almost always observed. These respiratory changes are measured by determining the oxygen uptake and carbon dioxide production, and tissue temperature changes due to evolution of heat of respiration by diseased in comparison to similar healthy tissues.

Weimer and Harter (1921) worked on the respiratory changes produced in the sweet potato by Rhizopus tritici. The

relative amount of carbon dioxide given off from the two halves of the same sweet potato, one of which was being decayed with R. tritici, was determined. It was found that the decaying half of the potato gave off a total of from 6.3 to 7.8 times as much carbon dioxide as the healthy half. Carbohydrate loss in the decayed sample was greater than that lost by respiration as measured by carbon dioxide evolution. They concluded that some of the carbohydrates were utilized in other processes such as the production of fungal materials, acids, alcohols, etc. Equally large increases in respiration in the tissues of Irish potatoes infected with Bacillus phytophthorus were reported by Eglits (1933). He also reported for this same disease a marked rise in temperature not only in the infected zone but also at points several centimeters away. Other workers have also reported temperature increases. Evans (1922) reported that oranges infected with Penicillium digitatum showed a distinct temperature increase and Gilman and Barren (1930) found that molds may markedly raise the temperature of stored grain but thought that this effect was due almost entirely if not entirely to the respiration of the molds.

Hellinga (1940) attempted to identify an active principle responsible for increased respiration of potato tuber tissue, obtained from extracts of the mycelium and

culture of Gibberella saubinetti, which causes a soft rot of the tubers. He found that mycelium extracts considerably increased oxygen uptake and carbon dioxide production and that smaller increases were produced by culture filtrates and by extracts of infected tubers. He concluded that the active principle might be pantothenic acid; however, this has not been confirmed.

Gretchushnikov (1953) reported on the toxins of the fungus Synchytrium endobioticum, the causal agent of potato wart. His studies with water and alcohol extracts of diseased potato tissue showed that under the influence of the toxins the enzymes which accelerate the mobilization of reserves and promote cell growth (formation of warts) are released from the protoplasm. The basic protoplasmic matter becomes more mobile, starch is changed to sugar, and proteins to amino acids. Conditions conducive to the development of the fungus and abnormal development of the cells of affected plants ensue. The growth of the cell is accompanied by increased respiration which, together with the growth processes, use up the plasmic matter and the cells of the wart tissue ultimately die.

Dorokhov (1939) grew tomato plants on various concentrations of Cladosporium fulvum culture filtrates or seed soaked in filtrates previous to planting and concluded from his

experiment that C. fulvum excretes into the nutrient medium toxins which suppress the development of the seed embryo and when introduced into the plant causes a partial or total poisoning of the host cells and breakup of the chlorophyll. Infection of leaves by the fungus causes a decreased rate of respiration.

Kuprevicz (1951) reported on the physiology of the diseased plant in relation to the general question of parasitism and stated that Wohltman potatoes slightly infected with Phytophthora infestans, the late blight fungus, showed an increased rate of respiration. He also noted that there was an insignificant increase in respiration in Victoria peas affected by Mycosphaerella pinoides, the causal agent of blight of peas.

Lutman (1934) studied the respiration rate of clean and scabby potato tubers through a storage period of 159 days. During the first month, the respiration rate of the scabby tubers was much higher than that of the clean tubers as the cork layers under the scab lesions were not so impervious to gasses as the skin of the uninjured ones. The rates tended to become equal during January and February but after sprouting began in March the clean tubers respired more than the scabby ones, as sprouting was delayed for sometime (7 to 10 days) by the scab lesions.

Parris (1941) working with bean leaves found that infection with anthracnose, Colletotrichum lindemuthianum, did not materially affect respiration. He explained this unexpected result by assuming that necrosed areas respire less, and non-necrosed areas, such as the margins of diseased spots, respire more than healthy tissue and under the existing conditions these two opposite phenomena counterbalanced each other.

Denny (1924) found that when lemons were exposed to very small concentrations of ethylene there was an increase in the respiration. Later Biale and Shepherd (1941) showed that the gaseous products of lemons infected with Penicillium digitatum caused a marked increase in the rate of carbon dioxide evolution and accelerated the color development of green lemons. Eminations from a single moldy lemon can produce these effects in 500 nearby fruits. The active agent is produced by P. digitatum growing on potato-dextrose broth agar medium as well as by moldy fruit. This volatile product of the rot fungus P. digitatum was later identified as ethylene by Young, Pratt and Biale (1951). Their work represents one of the clearest demonstrations of the chemical identity of a fungus toxin eliciting the symptoms of metabolic derangement in diseased tissues.

The preceding review indicates the effects of facultative parasites and their products on the respiration

of storage organs and other tissue. Equally marked rises in oxygen uptake and carbon dioxide production have been found in stems infected with obligate parasites. The effect on the host of mildew, rust and smut infections have been given considerable attention.

Long (1919) demonstrated that members of the genera Puccinia and Uromyces reduce the assimilation rate of oat and wheat leaves by approximately 30%, and Bailey and Gurjar (1919) found that wheat plants infected with P. graminis tritici exhibit a reduced rate of respiration.

Yarwood (1934) studied the effects of mildew and rust infection on dry weight and respiration of excised leaflets and found that Erysiphe graminis increased respiration 41% and Uromyces fallens increased respiration 123%. Dusting of healthy and mildewed leaflets with sulphur to kill the mildew indicated that the increased respiration of mildew infected leaflets was mainly due to a stimulation of the respiration of the host by toxins of the mildew rather than by respiration of the mildew fungus.

Pratt (1938) found that the respiration of leaves of wheat seedlings infected with powdery mildew, Erysiphe graminis tritici, rose rapidly and reached a maximum value of 2.5 to 3.0 times that of the controls in about 9 days. A

high rate of respiration was maintained for about a week and then it began to decline finally falling considerably below that of the controls. Only a small part of this increase of oxygen consumption was due to the mildew as removal of the mildew by sulphur dusting reduced oxygen uptake only a little.

Allen and Goddard (1938) working with the same host and fungus obtained almost identical results. They found that by mechanical removal of the mildew mycelium by differential poisoning of the mildew with sodium azide, and by measurement of the respiration of the intact mildew isolated on epidermal strips, that most of the increased respiration occured in the non-invaded mesophyll leaf tissues. Preliminary measurememts of the effect of the disease on photosynthesis indicates that the destruction of functional chlorophyll was subsequent to these changes in respiration. From these results they conjectured that toxic substances are produced by the mildew, and that the toxins diffuse across into the underlying host cells and these initiate metabolic changes which result in the increased respiration.

Allen (1942) divided the metabolic changes induced in wheat by powdery mildew infections into two phases. The first phase is characterized by an increase in the rate of oxidation of carbohydrate; by an increase in glucose, sucrose and starch; by a constant glucose respiration ratio; and by a

destruction of chlorophyll which is closely followed by a decrease in photosynthesis but not by a decrease in photosynthetic efficiency. The time required for these changes seems to correspond with the time required for the mildew toxins to spread into the leaf tissue because the first phase lasts considerably longer in lightly than in heavily infected leaves. The fact that the increase in respiration and the decrease in photosynthesis proceeds independently of each other during this phase even though they both reduce the normal supply of carbohydrates indicates that this is a period during which the mildew toxins are a major factor determining the trend of these two processes. Only after photosynthesis has been reduced to a very low level does the effect of carbohydrate starvation enter as a factor modifying the effect of the mildew toxins on the carbohydrate breakdown and the carbohydrate content of the leaves. The second phase is opened by a rather abrupt change in all the above mentioned relationships. The rate of respiration drops off rapidly and other substances than carbohydrates are being oxidized. Sucrose and starch disappear completely. The rate of photosynthesis continues to drop more rapidly than chlorophyll so that the photosynthetic efficiency falls below normal. During the early part of this phase the earliest attacked cells begin to form new chlorophyll which is however inactive in photosynthesis. Vegetative growth of the mildew slows down and it is

during this phase that green islands are formed. Allen followed the carbon balance per unit weight of leaf per day. Photosynthesis decreased from the first during a 12-day period from inoculation. Respiration increased for 7 days and then fell off. Carbohydrate increased for 6 days and then fell off. The carbohydrate available for transport decreased for 7 days and was a minus quantity thereafter. An abnormally large amount of starch and sucrose was found in mildewed leaves during the first 8 days after infection; thereafter both starch and sucrose disappeared completely. Observations made at an early stage of the disease would therefore lead to the conclusion that starch and sucrose had increased as a result of infection while if they were made later the opposite conclusion would be drawn. This feature might account for divergent results of workers who may have made their determination at different stages in the course of disease development. The accumulation of starch in the leaf tissue under the influence of the mildew is notable as the normal wheat leaf forms starch only in the guard cells.

Sempio (1950) reported that wheat plants infected with mildew showed an increased rate of respiration and glycolysis also increased during the first days of infection. In the following days, rate of respiration continued to rise rapidly until it attained (at the time conidia were differentiating) maximum values 3 to 4 times normal; glycolysis

decended rapidly to values below normal and continued to fall slowly until the end of the attack. He conjectured that the metabolic balance of the host is very important in determining its resistance and susceptability. In plants in a state of defence, the synthetic processes generally predominate over the breakdown processes while the contrary takes place in plants in a state of receptivity. Consequently, all ecological conditions of treatments which stimulate the respiratory activities, in disproportion to the photosynthetic or glycolytic activity, produce a state of predisposition and a special state of metabolic defence arises when the environmental conditions or treatments stimulate only, or preferably, the photosynthetic or glycolytic activity over the respiratory process.

Humphrey and Dufrenoy, (1944), discussed the theoretical basis of the respiration changes induced in the host by Puccinia coronata. The establishment of congenial host-parasite relationship entails a "decompensation" of the increased respiration suffered by the host cell so that it may be permitted to survive. If on the other hand the decompensation is so severe as to prove rapidly lethal, the rust fungus no longer behaves as a parasite but as a pathogen inducing necrotic spots characteristic of hypersensitivity. They consider that coacervation of phenolic compounds released

into the cell vacuole under the influence of the rust account for this decompensation of respiration as these compounds speed up respiration and coacervation would restrict their physiological activity.

Gottlieb and Garner, (1946), demonstrated that radioactive phosphorus accumulated in rusted parts of leaves and concentrated in the areas invaded by the parasite. From their own findings and those of others they formulated a picture of some of the physiological responses in obligate parasitism. As a reaction to the increased carbohydrate nutritive requirements of the parasite, sugars and phosphorus accumulate in the infected areas of the leaves. The augmented phosphorus supply might permit a greater activity of the phosphorylating enzymes of intermediary sugar metabolism. Thus, the increased amount of carbohydrates would be consumed and respiration consequently increased. Eventually, the demands upon the host cells are too drastic or some toxic action ensues and necrosis occurs.

Allen in (1953) and again in (1954) reviewed and summarized what is known of the effect of obligate parasitism on tissue respiration. Augmented respiration is almost a universal consequence of the infection of higher plants with obligate parasites. Much of the increased respiration is

contributed by the host cell and probably involves oxidation of carbohydrates during the period of vegetative growth of the parasite, since the respiratory quotient is close to 1.0 until the rate of respiration and the growth of the parasite begin to decline. Smaller increases or actual decreases in anaerobic carbon dioxide production accompany the increased respiration, and it is clear from Sempio's (1946) data that these changes are accompanied by an inhibition of the Pasteur effect. The Pasteur effect is the suppression of carbohydrate breakdown in air as compared with the rate of breakdown anaerobically, and has long been recognized as a wide spread property of aerobic cells. It is further known that the Pasteur reaction is inhibited by certain substances, with a resulting increase in aerobic carbon dioxide production and usually also in oxygen consumption. One of the most extensively studied compounds of this sort is 2, 4 dinitrophenol (DNP). Newcomb (1951) and Umbreit (1949) showed that low concentrations of DNP cause marked increases in the respiration of higher plant tissues. Hotchkiss (1949) studied the mode of action of DNP and showed that it inhibits the uptake of inorganic phosphate by intact cells and uncouples respiration from the energy requiring activities of the cell. Although the exact site of action of DNP is not definitely known, it appears likely that it acts upon energy rich phosphate substrate formed from oxidative phosphorylation, accelerating the re-

release of inorganic phosphate. Hunter (1951) states that if the removal of the aerobic inhibition is effected by an uncoupling action similar to that of 2,4 dinitrophenol, the resulting increase in intermediates would be confined to degradative processes and would be achieved by rapid regeneration of phosphate acceptors and of inorganic phosphate with loss of energy-rich phosphate. From the many indications that synthetic processes are also accelerated in plants infected with obligate parasites, it would seem that an uncoupling action is not the primary or exclusive cause of augmented respiration. The increase of phosphorus in the infection court, the stimulation of host cell growth by many obligate parasites and of synthesis of various substances by others, are evidence for an increase in the utilization of energy-rich phosphate as the cause of increased respiration. Greater hexokinase activity could have this effect as it does in many systems, including mitochondrial preparations from plants, and would lead to increased formation of glucose-6-phosphate, part of which might be diverted to starch synthesis. Opening other channels of utilization of energy-rich phosphate would also permit a more rapid aerobic breakdown of carbohydrate, accompanied in this case by an increased production of intermediates along another synthetic path. Sempio (1946) considers that the changes in

host metabolism after infection as well as those induced before infection, favor the further development of the parasite, and Cutter (1951) has recently outlined evidence in support of the view that phosphorylated intermediates of host metabolism are of critical importance in the development of obligate parasites. There are many signs which point in this direction, but whether the crucial substances lie along the path of carbohydrate breakdown or on the route of synthesis has not yet been clearly indicated. Since it is possible to block one path without simultaneously eliminating the other, this question could be put to experimental test.

## PECTIC ENZYMES IN PLANT DISEASE

The nomenclature of the pectic substances of plant tissues has been very confusing, and is still not fully cleared up. The literature contains well over fifty different terms for the various pectic substances and the meaning of these often overlap and may not be simply explained or, indeed, understood. The following classification of the pectic substances has been presented by Kertesz (1951).

"Pectic substances. Pectic substances is a group designation for those complex, colloidal carbohydrate derivitives which occur in, or are prepared from plants and contain a large proportion of anhydrogalacturonic acid units which are thought to exist in a chain-like combination. The carboxyl groups of polygalacturonic acids may be partly esterfied by methyl groups and partly or completely neutralized by one or more bases."

"Protopectin. The term protopectin is applied to the water-insoluble parent pectic substance which occurs in plants and which, upon restricted hydrolysis, yields pectinic acids."

"Pectinic Acids. The term pectinic acids is used for colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups. Pectinic acids,

under suitable conditions, are capable of forming gels (jellies) with sugar and acid or, if suitably low in methoxyl content, with certain metallic-ions. The salts of pectinic acids are either normal or acid pectinates."

"Pectin. The general term pectin designates these water-soluble pectinic acids of varying methyl ester content and degree of neutralization which are capable of forming gels with sugar and acid under suitable conditions."

"Pectic Acid. The term pectic acid is applied to pectic substances mostly of colloidal polygalacturonic acids and essentially free from methyl ester groups. The salts of pectic acid are either normal or acid pectates."

The enzymes concerned with the hydrolysis of the pectic substances are protopectinase, pectin-methylesterase, pectin-polygalacturonase and the newly discovered depolymerase.

Protopectinase is applied to the enzyme which hydrolyzes or dissolves protopectin with resultant separation of the plant cells from each other, a process usually spoken of as maceration. Thus far this enzyme has been demonstrated only by its action on plant tissues and a chemical definition of the reaction involved is impossible until the nature of protopectin itself is clarified.

Pectin-Methylesterase (pectase) is the enzyme which catalyzes the hydrolysis of the methyl ester group in pectinic acids and pectin with the subsequent formation of pectic acid and methyl alcohol.

Pectin-polygalacturonase (pectinase) is the enzyme that hydrolyzes the 1, 4 glycosidic linkages in the polygalacturonic acid skeleton of pectic or pectinic acids with the resulting formation of polygalacturonic acids of smaller molecular size and of (mono-) galacturonic acid.

The rigidity of plant tissue depends to a great extent on adhesion of cells and cell layers. This adhesion is, generally speaking, accomplished by the cementing effect of the intercellular pectic substances, especially those of the middle lamella. Most plant diseases related to the destruction of pectic constituents in the tissue result from the digestion of the cementing structures by the pectolytic enzymes which are excreted into the surrounding medium by various microorganisms. The digestion of the middle lamella results in reduction of the original structure into a mass of separate cells surrounded by a large amount of clear, light coloured liquid. This maceration of tissue is usually designated as "soft-rot". Many Plant Pathologists believe that the ability of fungi to secrete extracellular pectolytic enzymes which soften the tissue in advance of the hyphal tips

is one of the key biochemical features accounting for the pathogenicity of the various soft-rot organisms. On the other hand, it seems thet the secretion of pectolytic enzymes is not a common means of access of the pathogenic organisms to the tissues. In most cases, such organisms enter the plant only after mechanical injury, such as puncturing, cracking or bruising.

The dissolution action on the plant cell wall was first shown by DeBary (1886) for Sclerotinia libertiana. He demonstrated that drops which exuded from the sclerotia of this organism, as well as the juice expressed from decayed plant tissues, contained a substance of the nature of a ferment which was thermolabile and possessed the power of dissolving certain constituents of the cell walls so that coherence was lost. He observed differences in the activity of the boiled and unboiled extracts, but he was not able to determine whether the entire action of the extract was due to one or more substances. Similar results were obtained by Ward (1888) and Kissling (1889).

Nordhausen (1898) used extracts from old mycelium of a Botrytis species and came to the conclusion that the action on plant tissue was both enzymatic and toxic in nature.

Early work by Brown (1915) showed that there was a macerating principle in young and vigorously growing hyphae

of Botrytis cinerea. This extract actively decomposed a great variety of tissues. Heating for a short time at 60°- 70°C. destroyed the activity of the extract as regards both killing and macerating effects. Brown (1917) stated that both activities were due to one and the same substance, a cytolytic enzyme. Again Brown (1936) stated that investigations of several workers all point to the importance of cell wall composition in determining resistance and stresses the importance of the pectinase enzyme in parasitism.

Tribe (1951) reinvestigated the killing of cells by solutions containing protopectinases. Enzyme preparations were obtained from the soft-rotting pathogens Botrytis cinerea and Bacterium aroideae. He found that these preparations had three characteristic properties. They (1) decreased viscosity of pectin and pectate solutions (2) macerated parenchymatous tissue of higher plants and (3) killed cells of tissue so macerated. An unexpected result was obtained when plasmolyzed slices of tissue were placed in protopectinase preparations. It was found that maceration of the tissue was unhibited but there was a pronounced retardation of killing.

Fushtey (1953) repeated and extended the work done by Tribe. He showed that tissue slices, plasmolyzed and then carefully deplasmolyzed behaved like untreated slices; the

cells were resistant to killing during and after maceration only as long as they were plasmolyzed. He also observed that plasmolysis retarded the killing of cells by toxins such as oxalic acid and mecuric chloride.

Harter and Weimer (1921) working with culture and medium extracts (sweet potato decoction) of Rhizopus tritici, showed that the fungus produced a powerful intracellular and extracellular pectinase which dissolved the middle lamella of sweet potato disks so that the cells fell apart without the cells themselves undergoing any apparent mechanical alteration. Further studies using eleven species of Rhizopus, both parasitic and saprophytic species, showed that there was no correlation between the ability to produce pectinase and their ability to parasitize. All species were found to produce pectinase.

Pectin decomposition evidently is not the sole factor governing parasitism. For example, Harter and Weimer (1921) and (1923) showed that Rhizopus nigricans and R. artocarpi, severe rot inducing sweet potato parasites, were low in enzyme production whereas two non-parasitic forms Rhizopus chinensis, and R. microsporus, produced the pectin enzymes abundantly in solution though the mycelium itself was not rich in enzyme.

Davison and Willaman (1927) made a detailed study of the occurance in fungi of protopectinase. The enzyme was produced by Botrytis cinerea, and species of Rhizopus which are parasitic on sweet potatoes especially R. tritici. No protopectinase was found in Sclerotinia libertiana, and related forms.

Sideris (1924) found that Sclerotinia cinerea, the plum-rotting fungus, and Fusarium chronyophthoron form the enzyme protopectinase.

Spaulding (1911) observed that in the last stages of decay produced by Lenzites sepiaria the middle lamella disappeared and Zeller (1916) found that the same wood rotting fungus produced a substance called pectinase capable of dissolving the middle lamella of carrots and potato disks. Coherence of the tissue being entirely lost after 42 hours in an extract of the fungus powder.

Wood (1955) has reviewed the work carried on at the Imperial College and Technology, London, on types of protopectinase and other pectic enzymes secreted by plant pathogens. The protopectinase secreted by the following pathogens have been studied in some detail; Pythium debaryanum, Fusarium moniliforme, Botrytis cinerea, Verticillium dahliae, Sclerotium rolfsii and Bacterium aroideae. Verticillium dahliae

causes a typical wilt disease; the others produce a typical soft-rot of susceptible tissue.

A striking fact which has emerged from these investigations is that the properties of the protopectinase from each organism differ in one or more ways from those of the other organisms in the group.

With the exception of Pythium debaryanum the organisms produce protopectinase on relatively simple media. They differ, however, in their reaction to pectic substances in the culture solutions. Bacterium aroideae and Pythium debaryanum produce active solutions in the absence of pectic substances. Botrytis cinerea and Verticillium dahliae produce much more active solutions when they are present, Sclerotium rolfsii and Fusarium moniliforme produce protopectinase only in the presence of pectic substances.

The protopectinases from these six organisms differ widely in their pH optima. The enzyme from Sclerotium rolfsii has an optimum activity in the range 3.0 - 5.5; Botrytis cinerea has an optimum activity at 6.2 with a secondary high value at 2.6. The protopectinases from the other four organisms have suprisingly high optimal activity which are not easy to determine accurately but are in the range 8.5 - 9.5. Optima at such high values are difficult

to interpret owing to the de-esterfication of pectinic acids in alkaline media.

The protopectinases from these six organisms react quite differently to heat treatment. The most sensitive seems to be the enzyme from Pythium debaryanum which loses activity rapidly on exposure to temperatures in the range 40° - 50°C. and is almost completely inactivated after one minute at 55°C. The enzymes from Fusarium moniliforme and Botrytis cinerea are gradually inactivated as the temperature of exposure increases, little activity remaining after five minutes at 100°C. and 60°C. respectively. Bacterium aroideae shows anomalous behaviour; although activity is largely lost after short exposures at 70°C., there is a slight but definite increase in activity after exposure at 80°C. This effect is particularly pronounced for Sclerotium rolfsii. The enzyme from Verticillium dahliae also shows remarkable resistance to high temperatures; considerable activity remains after exposure for five minutes at a series of temperatures up to and including 100°C.

Considerable work has been done on the ability of soft-rot producing bacteria to ferment pectin. Jones (1911) determined that Bacillus carotovorus destroyed the middle lamella of soft vegetables and Smith (1903) reported this same ability for Bacterium campestre. Jones found that the

addition of carbohydrates to the medium of Bacillus carotovorus resulted in more intense production of pectolytic enzymes. On the other hand, the presence of cellular tissues in the culture had no marked effect, suggesting that the pectic constituents of tissue had at most an insignificant nutritive value for the organism.

Thornberry (1938) reported on the ability of fungal plant pathogens to produce pectase (pectin-methyl-esterase) on nutrient broth to which 10% pectin had been added. He used the ester substrate (half calcium salt of mono-methyl tartaric acid) and found that the extracts from cultures of the bacterial pathogens Phytomonas mori, P. tabaca, P. angulata and a bacterium from tobacco stem, possessed but slight activity. Those of Fusarium species that had been transferred several times on artificial media since isolation from diseased tobacco stems showed very slight or no activity, while freshly isolated cultures gave moderate hydrolysis. Three cultures of Sclerotium bataticola gave good tests, while those of Sclerotinia sclerotiorum and S. trifoliorum yielded but a fair amount of precipitate. Two specimens of a culture of Rhizoctonia that had been isolated for some time from diseased tobacco stems gave negative results. Three cultures of Thielaviopsis (535, 536 and 538) yielded positive but varying results. The culture 538, which possessed

moderate pectin-methylesterase activity, produced severe black-root rot of tobacco, while culture 535, with slight pectin-methylesterase activity, produced less severe rotting. The rotting capacity of culture 536, with marked enzyme activity, was not tested.

The existance of plant pathogenic forms responsible for the production of common scab of potatoes is an indication that host pectin decomposition may be a feature of the disease. Thaysen and Bunker (1927) showed that gums and pectin are highly suitable sources of carbohydrates for a large number of actinomycetes. Similar conclusions were drawn by Afanasiev (1937) who studied the ability of a number of parasitic and saprophytic species to decompose pectin. He states that the organisms first fermented pectin to pectic acid and the medium became more acid and then with continued growth the pectic acid was utilized and there was an increase in alkalinity. He concluded that there was no particular differences displayed by parasitic and saprophytic Actinomyces.

Lutman (1941) also showed that Actinomyces could utilize gum tragacanth, gum arabic and apple pectin as sources of carbohydrates when these were incorporated into the medium. Studies on the ability of Actinomyces to produce pectolytic enzymes have been few. As far as I can determine there has been no studies on the ability of Actinomyces to

produce these enzymes on various potato media.

By far the greatest amount of work has been devoted to pectinase (pectin-polygalacturonase), the enzyme responsible for complete rupture of the polymerized pectin molecule into its structural components. This enzyme is extremely widespread in fungi, both parasites and saprophytes. Waksman and Allen (1933) studied the decomposition of purified pectin and pectic acid by fungi and bacteria and observed that various microorganisms will attack pectin in different ways. Of the many organisms tested (mostly Penicillium, Aspergillus, and Fusarium species) the fungus Trichoderma proved to be very active in decomposing pectin. These authors observed that the pectin and pectic acid have to be decomposed before being utilized by the organisms.

Foster (1949) has reviewed the work reported by Harter and Weimer (1922), Gaumann and Bohni (1947) and Phaff (1947). These workers have shown that the pectinase enzyme complex is formed only when the organism grows on vegetable extract which of course contain pectin or in synthetic medium to which pectin has been added. In synthetic or in nitrogenous media in which pectin is absent these enzymes are not formed. It is clear then that these are adaptive enzymes, that is, formed only when the organism grows in the presence of the specific substrate. This situation is not entirely clear, however, and the situation

may be different in different fungi.

Gaumann and Bohni (1947) working with Botrytis cinerea found that pectin-polygalacturonase was a constitutive enzyme and pectin-methylesterase strongly adaptive. Extra-cellular enzyme formation was studied in each case. In a synthetic medium with glucose as the sole carbon source pectin-polygalacturonase activity was strong but there was practically no pectin-methylesterase formed. When pectin was present either in the presence or absence of glucose, abundant polygalacteronase formation was observed, reaching a maximum in four days. The methylesterase was not detected until the fifth day and reached its maximum in twenty-one days.

Phaff's studies (1947) with Penicillium chrysogenum indicate that extra-cellular polygalacturonase formation takes place only in response to the presence of certain specific substances in the medium: pectin, gum tragacanth, mucic acid, l-galacturonic acid and d-galacturonic acid.

In recent years the mechanisms by which certain pathogens cause their host plants to wilt have been much studied.

As a result of earlier studies with the tomato-wilt organism, Fusarium oxysporium var. lycopersici, Gaumann (1951) suggested that the peptide, lycomarasmin, which is

produced by the pathogen in old cultures, was in part responsible for the symptoms of wilt. However, work of Scheffer and Walker (1953), Gothoskar, Scheffer, Walker and Stahmann (1953) and Wienstead and Walker (1954), has provided evidence that lycomarasmin is not important as a cause of wilt in the living plant, but that many of the symptoms of disease are produced by pectin-esterase which is secreted by the pathogen on sterile moist warm wheat-bran. Solutions containing this enzyme caused typical vascular browning and wilt of the test plants, but, rather suprisingly, had little or no polygalacturonase activity. Pectin-methylesterase preparations from other microorganisms were also effective in producing typical wilt symptoms. The solutions used contained varying amounts of polygalacturonase, but there was no correlation between activity of enzyme and severity of wilt symptoms. As a result of these observations it was postulated that the production of pectin-esterase by the pathogen was an important cause of wilt symptoms in infected plants. The mechanism involved was not suggested. Wood (1955) states that these workers probably envisaged that the pectin-esterase acts upon high methoxy pectins of the xylem walls to give low methoxy pectinic acids or pectic acid which then react with polyvalent cations in the vascular stream to give gels which would interfere with the upward flow of water.

## MATERIALS AND METHODS - GENERAL

### SOURCES OF CULTURES

Fourteen different cultures of actinomycetes were used, three of which I isolated from scabby potatoes and the remainder were obtained from various sources in Canada and the United States. Nine of these cultures were parasitic strains of *Streptomyces scabies* and five of the cultures were saprophytic *Streptomyces* species.

Cultures L27, L38 and L45 were isolated from scabby Green Mountain potato tubers grown at Fredericton, N.B. These three strains differed morphologically when grown on Czapek's agar. All three strains produced severe scab infection.

Culture No. 10246 (*S. scabies*) was obtained from the American Type Culture Collection. The original isolation of this culture was made by W.H. Burkholder, Cornell University, and designated as strain N54.

Three parasitic strains of *S. scabies* S.429, S.449 and S.10 were obtained from L.A. Schaal, Division of Fruit and Vegetable Crops and Diseases, United States Department of Agriculture.

Parasitic cultures of *S. scabies* which were designated as $P_1$ and $P_3$ were obtained from the Department of

Bacteriology, Ontario Agriculture College, Guelph, Ontario.

Five saprophytic Streptomyces species S.106, S.122, S.129, S.403 and S.416 were also obtained from L.A. Schaal.

## THE ISOLATION OF ACTINOMYCETES FROM SCABBY POTATO AND SOIL WITH MINIMAL CONTAMINATION

The isolation of actinomycetes from scab-infected potatoes and from soil is often difficult because of numerous bacterial and fungal contaminants that overgrow the slower developing actinomycetes colonies. Shapovalov (1915), Taylor (1936), KenKnight and Muncie (1939) and Schaal (1940) proposed methods that reduce this difficulty when actinomycetes are being isolated from potatoes. With these methods some means of surface disinfection is employed, then under aseptic conditions the corky layer of a lesion is lifted, the underlying diseased area is removed and triturated in sterile water, and portions of the resultant suspension are diluted and plated. Isolations from soil are usually made by diluting the soil material and plating small aliquiots of this on selective media. Crook et al (1950) claimed that fewer contaminants appeared when sodium propionate was incorporated into the medium.

The above methods were found to be somewhat cumbersome and time consuming, consequently a more satisfactory

method of isolating these organisms was worked out. The following is a summary of the methods and results of this work.

## ISOLATION FROM SCAB INFECTED POTATOES

Preliminary experiments showed that, when actinomycetes were being isolated from scab-infected potatoes, most contaminants were eliminated by first treating a small portion of ground scabby tissue in a solution of phenol and water in proportion of 1:100 by volume. After 10 minutes in the phenol solution a small portion (1/25 ml.) of this treated material was placed in melted Czapek's agar and shaken before pouring it into a Petri dish. Experiments were then made with dilutions of phenol in water, ranging from 1:100 to 1:140 by volume, at dilution intervals of 10 ml.

Glucose-asparagine agar and Czapek's agar were used as the culturing media, which were adjusted to pH values of 6.0, 6.5, 7.0, and 7.5. These media were made aseptic by autoclaving for 15 minutes at 15 pounds pressure (121°C.).

The inoculum was prepared by grinding five gm. of peelings from scabby potato tubers in 100 ml. of distilled water for three minutes in a Waring blendor. One drop (1/25 ml.) of this material was then transferred to 10 ml. of the required phenol concentration, and to 10 ml. of distilled

water which served as a control. This suspension was thoroughly mixed and allowed to stand for 10 minutes; one drop was placed in 12 ml. of the melted medium at 45°C. The inoculated medium was then shaken and poured into a sterile Petri dish. After the plates had been incubated for seven days at 25° - 27°C., counts were made of the numbers of colonies of actinomycetes, fungi and bacteria that developed. In each experiment two plates were poured for each treatment, and the experiment was repeated six times.

The greatest numbers of actinomycetes were obtained on the two media at pH 6.5, and these results were presented in Table 1. Only occasional bacterial contaminants developed in any of the phenol treatments which also reduced the number of fungal contaminants. The numbers of actinomycetes were greatest after treatment with 1:140 phenol, and these decreased with increasing concentrations until finally a reduction, approximately 40%, occurred after treatment with 1:100 phenol. On Czapek's agar there was an increase in the number of actinomycetes over the control when the inoculum was treated with 1:110 to 1:140 phenol and on glucose-asparagine agar after treatment with 1:130 and 1:140 phenol. Only a few contaminants appeared on the media adjusted to pH 6.0, 7.0 and 7.5, but the development of actinomycetes was not as satisfactory as at pH 6.5; development at pH 7.0 and 7.5 was comparable to that at 6.5, but there were fewer

TABLE 1

THE EFFECT OF PHENOL IN REDUCING BACTERIAL AND FUNGAL CONTAMINANTS IN SCABBY POTATO INOCULUM

| Phenol Concentration | Mean count per plate on Czapek's agar at pH 6.5 (12 replications) | | | Mean count per plate on glucose-asparagine agar at pH 6.5 (12 replications) | | |
|---|---|---|---|---|---|---|
| | A | F | B | A | F | B |
| 1:100 | 108 | 0.4 | 0.2 | 123 | 0.5 | 0.0 |
| 1:110 | 192 | 1.4 | 0.2 | 221 | 1.0 | 0.0 |
| 1:120 | 227 | 1.1 | 0.1 | 206 | 0.0 | 1.0 |
| 1:130 | 238 | 2.3 | 0.1 | 257 | 2.0 | 0.5 |
| 1:140 | 277 | 1.3 | 0.1 | 309 | 1.8 | 0.1 |
| 0 | 190 | 8.0 | 34.0*+ | 251 | 3.0 | 31.0*+ |

A - Actinomycetes. F - Fungi. B - Bacteria.

*The + sign represents unrecorded pin point bacterial colonies.


Plate 1. Growth of actinomycetes, bacteria, and fungi on Czapek's agar, maintained at four pH levels, after the scabby potato inoculum had been treated with two phenol concentrations. Columns 1 to 4, medium adjusted to pH 6.0, 6.5, 7.0, and 7.5, respectively. Rows A, E, and F, phenol treatments 1:100, 1:140, and control, respectively.


Plate 2. The effect of five different phenol treatments on scabby potato inoculum plated on Czapek's agar at pH 6.5. A to F, phenol treatments 1:100, 1:110, 1:120, 1:130, 1:140 and control, respectively.

actinomycetes, whereas at pH 6.0 growth was slower than at pH 6.5, although there was a similar number of actinomycetes. Plate 1 shows the effect of two phenol treatments as compared to a control with the medium maintained at four pH values. Plate 2 shows the effect of treatment of scabby tissue with five concentrations of phenol previous to plating on Czapek's agar adjusted to pH 6.5.

A modification of this technique was particularly useful when actinomycetes were isolated from a single scab lesion. Isolated lesions were removed from Green Mountain and Irish Cobbler tubers and placed in a mortar containing 20 ml. of 1:140 phenol. The material was macerated with a pestle and the suspension allowed to stand for 10 minutes, then one drop was plated on Czapek's agar at pH 6.5. With this method a large number of different types of actinomycete colonies was obtained and these colonies were mostly free from contaminants.

## ISOLATION FROM SCAB INFECTED SOIL

The methods described above were also used, with slight modification, for the treatment of the inoculum obtained from soil. Two drops of a suspension containing five grams of soil in 100 ml. of water, were added to 10 ml. of phenol (1:100 to 1:140) and after 10 minutes one drop was plated on agar. Again the best results were obtained with

Czapek's and glucose-asparagine agar adjusted to pH 6.5, and with the 1:140 phenol treatment. These results are shown in Table 11. There was an increase compared to isolations from scabby potato ,in the number of bacterial contaminants on both media when inoculum was used from this source. However, when compared to the controls, the 1:140 phenol treatment reduced considerably the number of contaminants and permitted a larger number of actinomycetes to develop. Soil isolations differed from those obtained from scab-infected potatoes in that larger numbers of actinomycetes appeared on Czapek's agar than on glucose-asparagine agar.

## COMPARATIVE TESTS

A comparison was made of the method described and one in which contaminants are reduced by incorporating sodium propionate into the culture medium. Sodium propionate was incorporated into glucose-asparagine agar and Czapek's agar at the rate of 1:250 and the media adjusted to pH 6.0, 6.5, 7.0, and 7.5. The pH adjustments were made before autoclaving, and after autoclaving, and in one series there was no pH adjustment. The scabby potato inoculum was prepared as described previously and one drop of it was diluted in 10 ml. of distilled water. The two media were then inoculated with one drop of this suspension.

TABLE 11

THE EFFECT OF PHENOL IN REDUCING BACTERIAL AND FUNGAL CONTAMINANTS IN SOIL INOCULUM

| Phenol Concentration | Mean count per plate on Czapek's agar at pH 6.5 (12 replications) | | | Mean count per plate on glucose-asparagine agar at pH 6.5 (12 replications) | | |
|---|---|---|---|---|---|---|
| | A | F | B | A | F | B |
| 1:100 | 11 | 0.8 | 0.7 | 9 | 1.0 | 1.0 |
| 1:110 | 13 | 0.7 | 1.8 | 14 | 0.5 | 1.5 |
| 1:120 | 17 | 1.0 | 2.7 | 17 | 0.5 | 1.8 |
| 1:130 | 20 | 0.5 | 1.1 | 16 | 1.0 | 1.0 |
| 1:140 | 27 | 1.0 | 1.8 | 21 | 2.8 | 1.5 |
| 0 | 23 | 3.0 | 23.0*+ | 20 | 4.3 | 14.0*+ |

A- Actinomycetes. F. - Fungi. B - Bacteria.

*The + sign represents unrecorded pin point bacterial colonies.

When sodium propionate was incorporated into glucose-asparagine agar the results were unsatisfactory. No actinomycetes appeared on the medium at pH 6.0 and only a very few at pH 6.5, 7.0 and 7.5. When colonies did appear the development was slow and contaminants were numerous. When sodium propionate was added to Czapek's agar (pH adjusted before autoclaving) no actinomycetes appeared on the medium at pH 6.0 and only a few at pH 6.5. Satisfactory development occurred at pH 7.0 and 7.4. It thus appeared that the effectiveness of sodium propionate is influenced by the pH of the medium which, for best results, must be close to neutrality or slightly alkaline. In view of this, the comparative tests with the phenol methods were made with sodium propionate in Czapek's agar at pH 7.0. Phenol was used at a concentration of 1:140 with a treatment time of 10 minutes. The results of tests with soil inoculum and with potato inoculum are shown in Table 111. There were fewer actinomycetes and a greater number of contaminants with the sodium propionate than when the inoculum was treated with phenol.

## PATHOGENICITY TESTS

Tests were conducted to determine the pathogenicity of the cultures isolated from scabby potatoes and those obtained from investigators in Canada and the United States. The three methods employed were: a. greenhouse tests of pathogenicity, b. pathogenicity of axillary tubers on stem-leaf cuttings, and c. pathogenicity on detached potato tubers.

TABLE 111

COMPARATIVE EFFECTS OF SODIUM PROPIONATE AND PHENOL IN REDUCING FUNGAL AND BACTERIAL CONTAMINANTS WHEN ISOLATING ACTINOMYCETES

(Inoculum plated on Czapek's agar at pH 7.0)

| | Scabby Potato Inoculum | | | Soil Inoculum | | |
|---|---|---|---|---|---|---|
| Organism | Phenol 1:140 | Sodium Propionate 1:250 | Control | Phenol 1:140 | Sodium Propionate 1:250 | Control |
| Actinomycetes | 172 | 154 | 131 | 17 | 15 | 15 |
| Bacteria | 0 | 45*+ | 57*+ | 1 | 12 | 20*+ |
| Fungi | 1 | 3 | 2 | 8 | 9 | 19 |

* The + sign represents unrecorded pin point bacterial colonies.

a. Greenhouse Tests of Pathogenicity. This method was similar to that described by Schaal (1944). Green Mountain seed pieces were treated with a 1 to 1000 mercuric chloride solution for 1½ hours, then washed under tap water for 10 minutes. Preliminary tests showed that the tap water contained no actinomycetes. The soil was steam sterilized by placing a two-inch layer of soil in a small wooden flat and autoclaving it for two hours at 15 pounds pressure (121°C.) Seven-inch clay pots were sterilized by placing them in a 1-40 formaldehyde solution and leaving them overnight. The bench on which the pots were to sit was sprayed with a three percent formaldehyde solution and separated from the rest of the greenhouse with a screen made from unbleached cotton. The pots were partly filled with sterile soil and each planted with a seed piece. The seed piece was then covered with a shallow layer of soil which was inoculated by pouring over it 200 ml. of water suspension of mycelium and spores which was prepared by scraping the growth from three Petri dish cultures of the test organism. In all cases the pot was filled with soil to within one inch from the top. Each culture was tested in triplicate and the inoculated pots were separated from one another by a Green Mountain control. The pots were watered daily with a small stream of water in order to avoid splashing. Scab readings were made 110 days after planting.

The pathogenicity of 11 cultures of Streptomyces scabies were tested by this method. The results are presented in Table IV.

The strains L27, L45, and L38 of S. scabies isolated from Green Mountain potatoes produced severe scab.

The three cultures S.429, S.449, and S.10 were allegedly parasitic strains of S. scabies received from

TABLE IV

THE PATHOGENICITY OF 11 CULTURES OF STREPTOMYCES SPECIES ON GREEN MOUNTAIN POTATO TUBERS

| CULTURE NO. | SCAB REACTION |
|---|---|
| L27 | Severe |
| L45 | " |
| L38 | " |
| S.429 | Negative |
| S.449 | " |
| S.10 | " |
| S.106 | " |
| S.122 | " |
| S.129 | " |
| S.403 | " |
| S.416 | " |

L.A. Schaal, but they did not produce scab on Green Mountain potatoes in these tests. These cultures will be referred to in this thesis as avirulent strains of S. scabies.

The saprophytic Streptomyces species S.106, S.122, S.129, S.403, and S.416 did not produce any reaction on the potato tubers.

b. Pathogenicity on Axillary Tubers on Green Mountain Stem-Leaf Cuttings. This method was similar to that described by Busch and Gilpatrick (1950). Stem-leaf cuttings were made from Green Mountain plants that were in bud or blooming. These were obtained by cutting the stem just above the axil of a leaf and about 5 mm. below it so that the bud in the axil was retained intact. These cuttings were placed in wet, sterile vermiculite where they developed, in two to three weeks, a small axillary tuber 5-18 mm. in diameter. The leaf-cutting with its axillary tuber was removed from the vermiculite and the tuber inoculated by dipping it in a mycelium-spore suspension of the test organism. The cutting with its axillary tuber was replanted in a three-inch pot containing moist vermiculite maintained at approximately 90% of its moisture holding capacity. Readings were made 7-14 days after inoculations. Five cultures were tested by this method and the results are shown in Table V. All five isolates showed definite pathogenicity.

TABLE V

THE PATHOGENICITY OF FIVE CULTURES OF STREPTOMYCES SCABIES ON AXILLARY TUBERS ON GREEN MOUNTAIN STEM-LEAF CUTTINGS

| CULTURE NO. | SCAB REACTION |
|---|---|
| 10246 | Slight |
| $P_1$ | Moderate |
| $P_3$ | " |
| L27 | Severe |
| L45 | " |

c. Pathogenicity on Detached Green Mountain Potato Tubers. Small axillary tubers from stem-leaf cuttings were obtained as described in the preceeding section. Tubers 5-18 mm. in diameter were detached, washed in tap water and immediately inoculated either by being revolved while in contact with the surface growth of a heavy sporulating culture or by being dipped in a mycelium-spore suspension of the test culture of the organism. Three parasitic and three saprophytic cultures were tested. After inoculation the tubers were placed in Petri dishes and covered with sterile fine-grade moist vermiculite. Scab readings were made 7-14 days after inoculation. The results of this test are presented in Table VI.

TABLE VI

PATHOGENICITY OF SIX STREPTOMYCES SPECIES ON DETACHED AXILLARY GREEN MOUNTAIN POTATO TUBERS

| CULTURE NO. | SCAB REACTION |
|---|---|
| L27 | Severe |
| L45 | " |
| L38 | " |
| S.122 | Negative |
| S.106 | " |
| S.403 | " |

The small tubers, inoculated with the three parasitic cultures developed initial infections at the lenticels after two days and eruptive scab lesions after a week's incubation. Microscopic examination of the scab lesions showed numerous strands of the fine mycelium, characteristic of the scab organism. Isolations from the lesions gave cultures morphologically similar to those used for the inoculations. Tubers inoculated with the non-parasitic cultures failed to show any symptoms after four weeks.

The foregoing pathogenicity tests showed that the isolates designated as L27, L38 and L45 are severe pathogens causing almost 100% scab on Green Mountain Potato tubers. Isolates $P_1$ and $P_3$ produced a moderate amount of scab, while isolate No. 10246 produced only a slight amount of scab on the potato tubers. No differences in the type of scab lesions produced by the above isolates were noted. Although isolates S.429, S.449, and S.10 were allegedly parasitic they did not produce any scab on Green Mountain tubers. The saprophytic isolates did not have any perceptible effect on the potato tubers.

The greenhouse method of testing for pathogenicity is a standard one and has been used extensively in the past by workers in this field. The stem-leaf cutting method and the detached tuber method are relatively new and are still in the

experimental stage. However, I found the latter two methods to be of value in testing for pathogenicity, because they save time and work and results are obtained over a shorter period of time. Testing the pathogenicity of isolates by the detached tuber method has the added advantage of giving the worker greater opportunity of exercising control over the temperature and humidity at which the tests are to be conducted.

## RESPIRATION STUDIES

### ORGANISMS USED

The purpose of this study was to compare the effect, on potato tissue respiration, of extracts of media on which parasitic and saprophytic streptomycetes had grown. The following pathogenic isolates were selected because of a difference in their virulence; strain No. 10246 was slightly virulent, strains $P_1$ and $P_2$ were moderately virulent, while strain L27 was strongly virulent. The saprophytic species selected were S.403 and S.416. Although six isolates were used in the respiration studies, most of the work was done with media extracts of the slightly virulent strain of S. scabies No. 10246.

### CULTURAL METHODS EMPLOYED

Czapek's agar, Czapek's solution and potato decoction liquid medium were the three media employed for growing the various Streptomyces species.

Czapek's agar was prepared and adjusted to pH 6.7, and 100 ml. of it were placed in Roux culture flasks which were plugged with cotton wool and sterilized for 15 minutes at 15 pounds pressure (121°C.). The flasks were then laid on a bench and the medium allowed to cool and set. The surface of

the medium was inoculated with 5 ml. of mycelium-spore suspension of the test organism and the flask was rotated until the entire surface of the medium had been covered. The excess inoculum was poured off, the cotton plug replaced and the culture was incubated at 27°C.

Czapek's solution was prepared and adjusted to pH 6.7. Seventy-five ml. were placed in 500 ml. flasks and sterilized for 15 minutes at 15 pounds pressure (121°C.). The flasks were allowed to cool and then inoculated with 5 ml. of mycelium-spore suspension of the test organism. These cultures were grown as shake cultures on a shaking machine having 70 oscillations per minute. They were grown at a temperature of 25°-28°C.

The potato decoction was prepared by steaming 250 gm. of Green Mountain potato tuber tissue in one liter of distilled water for 45 minutes. The resulting liquid was filtered through four layers of cheese cloth and the volume made up to one liter. Fifteen ml. were placed in 125 ml. Erlenmeyer flasks which were plugged and then sterilized for 15 minutes at 15 pounds pressure (121°C). When the medium had cooled it was inoculated with one ml. of a suspension of the test organism. Cultures were incubated at a temperature of 27°C. Controls for the above three culturing media were prepared by adding a similar amount of water to uninoculated slices.

## PREPARATION OF INOCULUM

The stock cultures of all the organisms used in the respiration studies were grown on Czapek's agar. The inoculum was prepared by flooding the surface growth of three test tube slant cultures of the test organism with 10 ml. of sterile distilled water. The surface of the culture was scraped and the resultant suspension was poured into a 125 ml. flask which contained 20 ml. of sterile water. This suspension was used for the inoculations of the three media.

## PREPARATION OF MEDIUM EXTRACTS

Extracts from Czapek's agar, Czapek's solution and potato decoction liquid medium were made after 3, 5, 7, 9, 11, 13, and 15 days of growth. In preparing the extracts from Czapek's agar 30 ml. of sterile distilled water were added to a culture. The flask was plugged with a cork and placed on a shaking machine and vigorously shaken for one hour. The resultant material was strained through four layers of cheese cloth and filtered through a glass frittered filter. The filtrate was passed through a sterile L5 or L7 Pasteur-Chamberlain filter candle and collected in a sterile evacuated flask.

Extracts from Czapek's solution and the potato decoction solution medium were prepared by passing the media

first through a glass frittered filter and then through a clean sterile L5 or L7 Pasteur-Chamberlain filter candle and collected in sterile evacuated flasks.

The extracts were used on the same day in which they were prepared.

Extracts to be used as controls were prepared from the three media which had received no inoculum.

## PREPARATION OF TISSUE

The tissue used for the respiration studies was obtained from Green Mountain potato tubers. These tubers were kept in storage, maintained at approximately 5°C., until 24 hours before using when they were removed and placed in the laboratory where the temperature was between 22°- 26°C. The tubers were peeled, stem and rose ends discarded and the remaining portion cut into cross-sectional slices about 2 cm. thick. A cylinder of tissue was then cut from the clear central portion of the internal medullary with a stainless steel cork-borer, 5.6 mm. in diameter. These cylinders were placed in a Petri dish and washed with tap water. The cylinder of tissue was finally cut into disks 0.5 mm. thick, with a Bauch and Lomb hand microtome and a clean sharp razor blade. These were placed in tap water in a Petri dish and

examined under a disecting microscope. Any disks showing vascular tissue present were discarded. After the required number of suitable disks had been obtained they were washed four times in tap water and three times in distilled water and then left in distilled water for one hour before using.

## MANOMETRIC METHODS

The Warburg constant volume respirometer was used for the manometric measurements. The "direct method" of Warburg was employed to determine the oxygen taken up by living tissue. The liberated carbon dioxide was absorbed by alkali during the determinations. This method is described by Umbreit, Burris and Stauffer (1951).

The Warburg manometers and flasks were calibrated by the mercury-water method described by Umbreit, Burris and Stauffer (1951).

The reaction of manometer flask, which had a volume of approximately 15 ml., consisted of a center well and one side arm equipped with a ground glass venting plug. The flasks were cleaned after each run by washing overnight in a potassium dichromate solution, and thoroughly rinsing with tap water and distilled water. They were then placed in an oven, maintained at 70°C. to dry.

The following was the procedure in setting up the manometer flasks for the respiration studies. To the main chamber of a manometer flask was added two ml. of a M/15 phosphate buffer at pH 6.7. To this were added 20 disks of potato tissue immediately after being removed from distilled water and blotted to absorb the excess water. A rolled strip (10 mm. X 30 mm.) of Watman No. 1 filter paper was placed in the small center well of the flask to which was added 0.2 ml. of a 20% potassium hydroxide solution.

After the manometer flask was set up in the manner described one ml. of the medium extract, on which the organism had grown, was added to the main chamber of a flask. The control was set up in the same manner with the exception that an extract prepared from the uninoculated medium was added. A thermobarometer was prepared by adding only 3.2 ml. of the M/15 phosphate buffer to one manometer.

The flasks were placed on their respective manometers and the Warburg set to oscilate at 98 to 104 strokes per minute. The temperature of the bath was maintained at 27°C. The flasks were allowed to shake for 15 minutes before closing the stopcock of the manometers. The solution in the manometers was zeroed at 150 mm. just before the stopcocks were closed.

Readings were made every 15 minutes by bringing the fluid in the closed arm of the manometer back to the 150 mm.

mark and then reading the level of the fluid in the open arm of the manometer. The uptake of oxygen, in mm., was calculated by subtracting the initial reading from all subsequent readings of the manometer. The thermobarometer correction was obtained by subtracting the initial reading from all subsequent readings. The actual change in mm. was determined by subtracting the corrected thermobarometer reading from the corrected uptake reading of the manometer. This value multiplied by the flask constant for the conditions employed gave the $\mu$l of oxygen taken up by the tissue in the reaction flask.

When the respiration run was completed the potato tissue was removed from each of the manometer flasks and the excess liquid on the tissue was blotted with bibulous paper. The tissue was placed in a small vial (approximately 2 ml.) which had been previously tared. The vials were placed in an oven at 90°C. and the tissue was allowed to dry overnight. The vial and the dried material were placed in a desicator to cool and then weighed. The weight of the tissue was determined by subtracting the total weight from the weight of the vial. From these weights the amount of oxygen consumed per mg. of dry weight of tissue was determined.

## RESULTS OF RESPIRATION STUDIES

A large portion of the respiration studies was done with extracts from media on which the parasitic strain of S. scabies No. 10246, had grown. The results of this study are presented first.

Strain No. 10246 grew well on Czapek's agar and in shake cultures of Czapek's solution. As the organism grew on these media it caused the media to become alkaline and often the pH of the media was raised to pH 8.6. This fact confirms a number of reports that actinomycetes have a tendency to make alkaline the medium on which they are growing.

Treatment of potato tuber tissue with extracts from Czapek's agar, on which the organism had grown for 3, 5, 7, 9, and 11 days, caused a reduction in respiration. This effect is shown in graph form in Fig. 1 and 11. The symbol $Q_{O_2}$ represents the amount of oxygen taken in per mg. dry weight of tissue. Medium extracts on which the organism had grown for three days effected only a 0.6% reduction in the respiration rate of potato tissue. The greatest reduction in tissue respiration was effected with medium extracts after 5, 7, and 9 days growth of the organism. This is clearly shown in Table V11, and in Fig.1, graphs B, C, and D.

TABLE V11

EFFECT ON POTATO TISSUE RESPIRATION OF EXTRACTS FROM CZAPEK'S AGAR ON WHICH STREPTOMYCES SCABIES NO.10246 HAD GROWN FOR VARIOUS PERIODS

| NUMBER OF DAYS OF GROWTH | EFFECT UPON $O_2$ UPTAKE |
|---|---|
| 3 | 0.6% Reduction |
| 5 | 58.5% " |
| 7 | 57.6% " |
| 9 | 51.5% " |
| 11 | 20.0% " |
| 12 | None |
| 13 | None |

The data presented in Fig.11 graph E. shows that the extract from Czapek's agar on which the organism had grown for 11 days had less effect on tissue respiration than medium extracts from 5, 7, and 9-day old cultures. Medium extracts from 3-day old cultures effected a slight reduction in the respiration of potato tissue. There was no effect by medium extracts of 12 and 13-day old cultures. This particular experiment shows that the greatest depression in potato tissue respiration was effected by treatment with a medium extract from a 5-day old culture. However, this was not always the case; the greatest reduction was sometimes effected with medium extracts from 7-day old cultures.

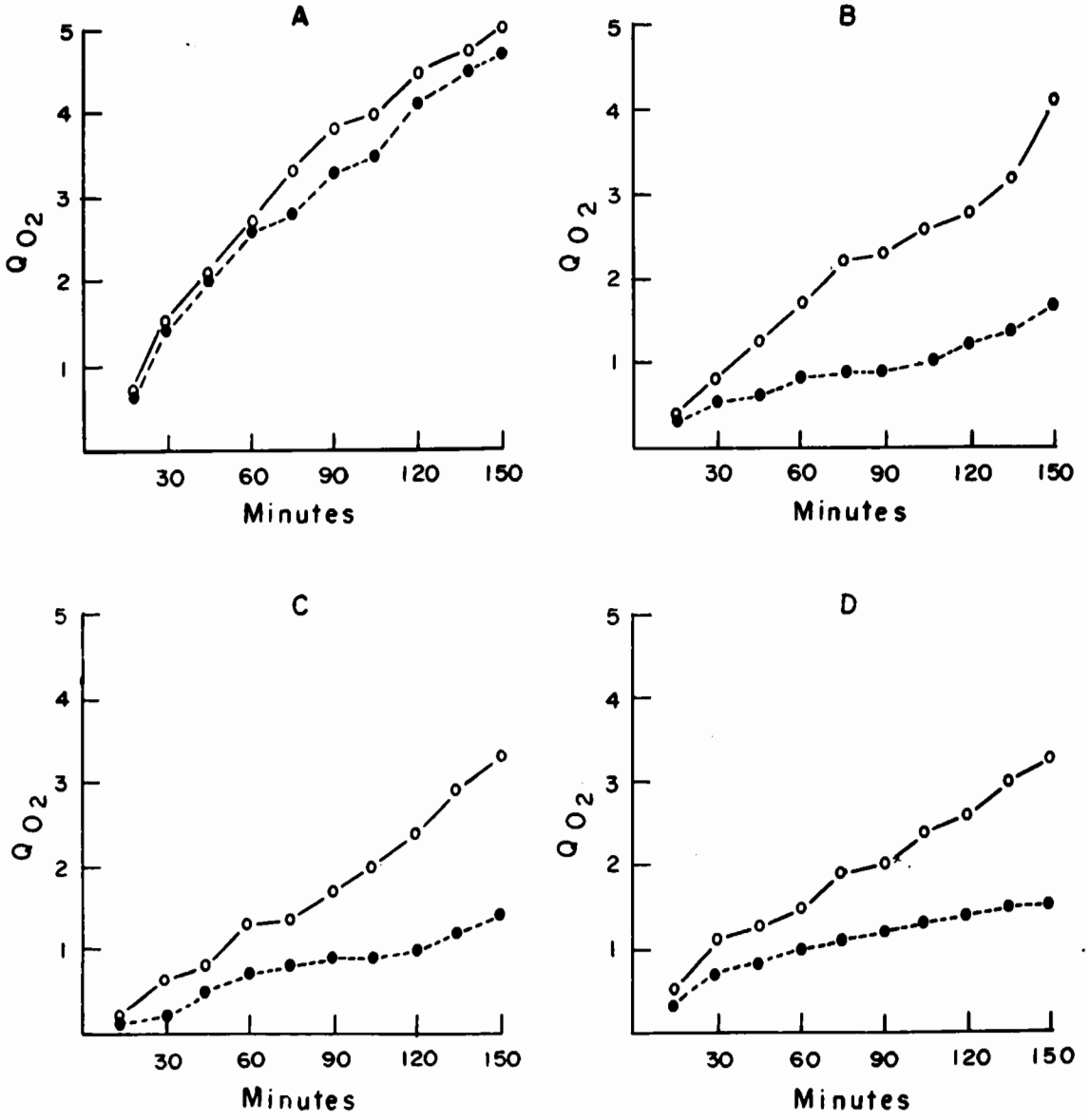


Figure 1. Effect of Streptomyces scabies, No. 10246 culture extract on the $O_2$ uptake of tissue from potato tubers. A, B, C and D are the curves given by extracts of Czapek's agar on which S. scabies had grown for 3, 5, 7 and 9 days respectively.

•----• Extract o——o Control

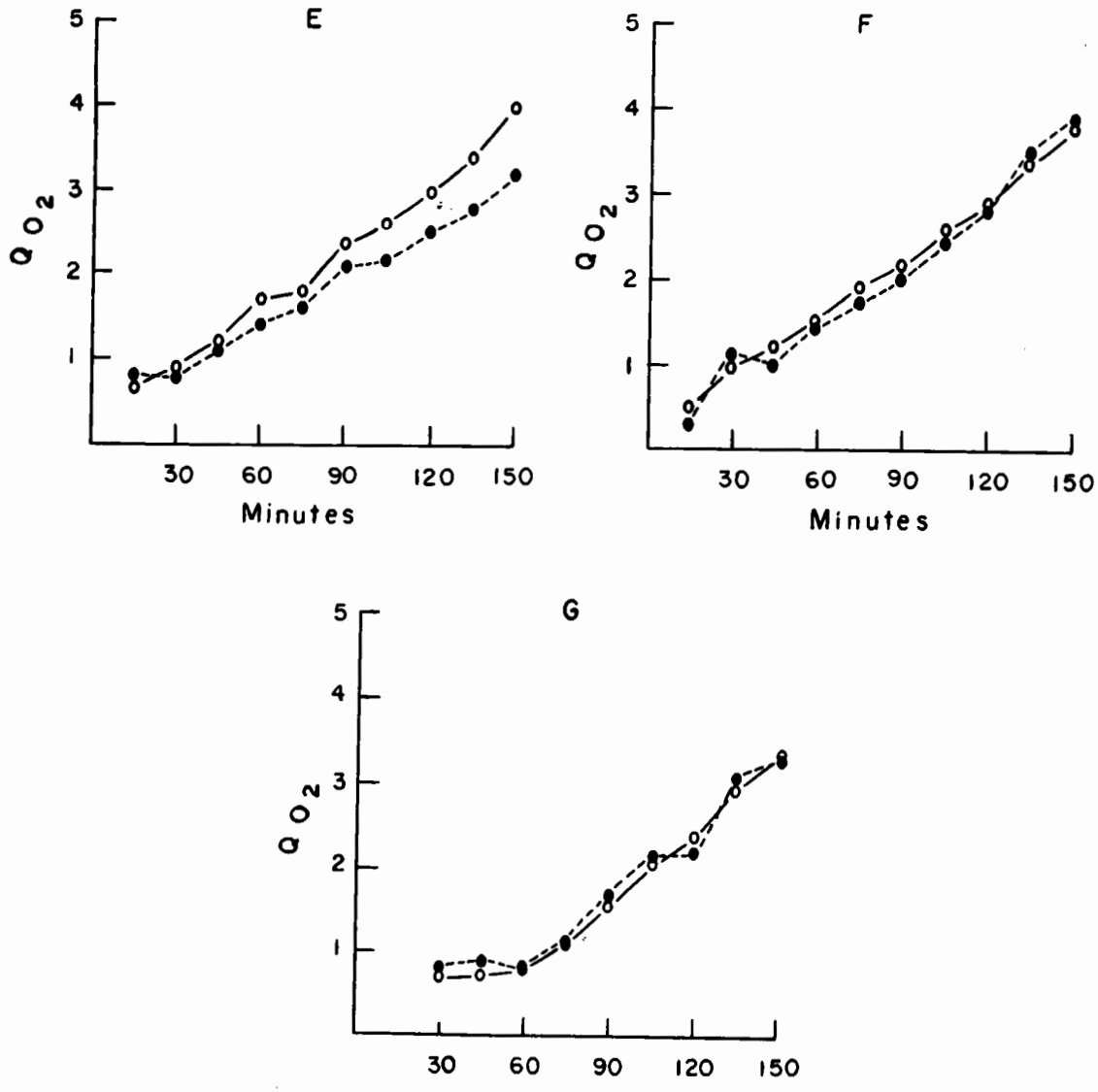


Figure 11. Effect of Streptomyces scabies No. 10246, culture extract on the $O_2$ uptake of tissue from potato tubers. E and F are the curves for extracts of Czapek's agar on which S. scabies had grown for 11 and 13 days respectively. G, effect of heat (10 minutes at 60 C.) on an extract from a 7-day old culture.

•----• Extract o——o Control

When extracts from 3, 5, 6, 9, and 11-day old cultures were heated to 60°C. for 10 minutes they lost their ability to inhibit potato tissue respiration. Graph G. Fig. 11 shows that heating the medium extracts from 7-day old cultures had no effect on potato tissue respiration. This result indicated that the respiratory inhibitory substance was proteinaceous in nature.

Extracts of Czapek's agar on which the parasitic strain No. 10246 had grown, fluoresced in the presence of ultraviolet light. The fluorescent intensity was measured to determine whether a correlation existed between the fluorescence of the extracts and the effect of these extracts on potato tissue respiration. Measurements were made with a Coleman Universal Spectrophotometer, Model 14, equipped with an Ultraviolet Illuminator and a UV-1 filter. The results are presented in Table VIII.

The intensity of the fluorescence of the extracts increased with age of the culture. There was no correlation between the amount of fluorescence of the extracts and the effect of these extracts on potato tissue respiration.

S. scabies No. 10246 was also grown on Czapek's solution in shake cultures. The effects of medium extracts of cultures of varying ages on potato tissue respiration are presented in Table IX.

TABLE V111

THE FLUORESCENT INTENSITY OF EXTRACTS OF CZAPEK'S AGAR ON WHICH STREPTOMYCES SCABIES ISOLATE NO. 10246 HAD GROWN FOR VARYING PERIODS

| NUMBER OF DAYS GROWTH | FLUOROMETRIC READINGS |
|---|---|
| 3 | 4 |
| 5 | 7 |
| 7 | 11 |
| 9 | 15 |
| 11 | 17 |
| 13 | 17 |
| 15 | 19 |
| 17 | 16 |
| 19 | 20 |

TABLE 1X

EFFECT ON POTATO TISSUE RESPIRATION OF EXTRACTS FROM SHAKE CULTURES (CZAPEK'S SOLUTION) ON WHICH STREPTOMYCES SCABIES NO. 10246 HAD GROWN

| NO. OF DAYS GROWTH | EFFECT ON $O_2$ UPTAKE |
|---|---|
| 3 | None |
| 5 | 71.7% Reduction |
| 7 | 74.5% " |
| 9 | 55.0% " |
| 11 | 37.0% " |
| 13 | 13.0% Increase |
| 15 | 9.7% " |

There was a greater inhibition of respiration when potato tissue was treated with extracts from shake cultures 5, 7, 9 and 11-days old in Czapek's solution than with extracts from corresponding cultures on Czapek's agar. A 74.5% decrease in respiration was produced with extracts from 7-day old shake cultures while the corresponding extract from the solid medium gave a reduction of 57.6% (See Table V11). Medium extracts from 13 and 15-day old cultures caused some increase in the respiration of potato tissue while extracts from 3-day old cultures had no effect on tissue respiration.

Two other parasitic strains of S. scabies designated as $P_1$ and $P_3$ were also grown on Czapek's agar. When potato tissue was treated with medium extracts from these cultures respiration of the tissue was stimulated. The effect on the respiration of tissue treated with culture extracts of the parasitic strain $P_1$ is shown in Table X.

TABLE X

EFFECT ON POTATO TISSUE RESPIRATION OF EXTRACTS FROM CZAPEK'S AGAR ON WHICH STREPTOMYCES SCABIES STRAIN $P_1$ HAD GROWN

| NO. OF DAYS GROWTH | EFFECT ON $O_2$ UPTAKE |
|---|---|
| 3 | 30.0% Increase |
| 5 | 29.0% " |
| 7 | 11.3% " |
| 9 | 16.0% " |
| 11 | 14.0% " |
| 13 | 23.6% " |

Similar results were obtained when potato tissue was treated with medium extracts from cultures of S. scabies strain $P_3$. The greatest increase in respiration was with extracts from the medium on which the organism had grown for 9 days. Respiration was increased 47.3%.

The parasitic strain of S. scabies L27 was grown on potato decoction medium and extracts were prepared from the medium after various periods of growth. The effect of these extracts on the respiration of potato tissue were determined and the results are presented in Table Xl.

TABLE Xl

THE EFFECT ON RESPIRATION OF POTATO TISSUE OF EXTRACTS FROM POTATO DECOCTION MEDIUM ON WHICH STREPTOMYCES SCABIES L27 HAD GROWN

| AGE OF CULTURE | EFFECT ON $O_2$ UPTAKE |
|---|---|
| 3 days | 7.4% Reduction |
| 6 " | None |
| 8 " | 8.8% Increase |
| 10 " | 9.4% " |

Extracts from a 3-day old culture had some inhibitory effect on the respiration of potato tissue while extracts from a 6-day old culture had no effect. Medium extracts from 8 and 10-day old cultures effected an increase in respiration.

Experiments with extracts from potato decoction medium on which two saprophytic species S.416 and S.116 had grown, showed that respiration of potato tissue was increased with extracts from 7-day old cultures of S.416 and decreased with extracts from corresponding cultures of S.116.

## PECTOLYTIC ENZYMES

This study was undertaken to determine if a number of Streptomyces species could produce some or all of the pectolytic enzymes and whether the enzymes produced would be an aid in separating pathogenic from non-pathogenic forms as well as and aid in elucidating the host-parasite relationship.

### ORGANISMS USED

Four virulent and three avirulent strains of Streptomyces scabies and five saprophytic Streptomyces species were used for the enzyme studies. These were as follows:

| Parasitic Spp. | Saprophytic Spp. |
|---|---|
| L27 Virulent | S.106 |
| L38 " | S.122 |
| L45 " | S.129 |
| No.10246 " | S.403 |
| S.10 Avirulent | S.416 |
| S.429 " | |
| S.449 " | |

### MEDIA USED

Two potato media were employed in these studies to determine if actinomycetes could produce pectolytic enzymes when

potato tissue or decoction was used as the substrate. These media were designated as (a) sliced potato tissue medium and (b) potato decoction medium.

(a) Sliced Potato Tissue Medium. A medium sized Green Mountain potato tuber was washed, dried. The surface of the tuber was sterilized by placing it in an acidified 1 to 300 mecuric chloride solution for five minutes. The tuber was then washed with sterile water and placed under sterile paper towels.

Under aseptic conditions the eye and stem-end of the tuber were cut away and the remainder of the tuber was cut into slices 10 mm. thick. The periderm layer of each slice was removed and the slice was measured with a sterile ruler and cut into cubes approximately one cm. square. The cube was then cut into 10 slices and these were transferred to a sterile 125 ml. Erlenmeyer flask which contained 10 ml. of sterile distilled water. The tissue slices were inoculated with one ml. of a suspension of the test organism and the flask was placed in an incubator at a temperature of 28°C. Controls were prepared by adding one ml. of sterile water to the tissue instead of the suspension of the organism.

(b) Potato Decoction Medium. Five hundred gm. of sliced unpeeled Green Mountain potato tubers were steamed for

fourty-five minutes in one liter of distilled water. The resulting liquid was filtered through four layers of cheese cloth and the volume made up to one liter. Potassium dihydrogen phosphate was added as a buffer at the rate of one gm. per liter. The pH of the medium was 6.5. Fifteen milliliters of the medium were placed in 125 ml. Erlenmeyer flasks which were plugged and sterilized for 15 minutes at 15 pounds pressure (121°C.). The flasks were cooled and the medium inoculated with one ml. of a suspension of the test organism. Controls were prepared by adding one ml. of water to the medium instead of the suspension of the test organism.

## PREPARATION OF THE INOCULUM

All stock cultures were grown on Czapek's agar with the exception of the parasitic culture L45 which grew best on glucose-asparagine agar. The inoculum for the potato media was prepared by flooding the surface growth of each of three test tube slant cultures of the test organism with 10 ml. of sterile distilled water and scraping the surface. The resultant suspension was poured into a 125 ml. sterile Erlenmeyer flask containing 20 ml. of water. This gave a final volume of 50 ml. of inoculum. One ml. of this was used to inoculate each flask.

## PREPARATION OF CULTURE EXTRACTS FROM POTATO MEDIA

After the required period of growth the liquid of the culutres was strained through four layers of cheese cloth. In the case of the sliced potato tissue medium the material was squeezed through the cheese cloth in order to remove the liquid from the potato tissue. The strained extracts were passed through a sterilized Seitz filter and the liquid collected in sterile flasks. This extract was tested for enzyme activity.

## RESULTS OF PECTOLYTIC ENZYME STUDIES

The production of pectolytic enzymes, on two potato media, by a number of Streptomyces species was studied. The results are presented in two sections as follows: (a) protopectinase and (b) other pectolytic enzymes.

(a) Protopectinase. The lack of knowledge about protopectin (the substrate) is well reflected in the methods of estimating protopectinase activity. Quantitatively, protopectinase is measured by the progress of maceration. This method was used to determine the presence of protopectinase in the cultures of S. scabies employed.

Standard slices of potato tissue, 0.5 mm. thick, 5.0 mm. wide, and 20 mm. long, were used in the enzyme tests.

Three slices were added to 10 ml. of the enzyme solution (extract) in a test tube. A thin layer of toluene was added to reduce the growth of aerobes. The test tubes were placed in a water bath maintained at 35°C. and the time required for complete maceration of the tissue was determined. A tissue slice was assumed to be completely macerated when no perceptible force was needed to pull it apart.

A number of parasitic Streptomyces species as well as a number of saprophytic Streptomyces species produced the tissue macerating enzyme protopectinase. It was found that one parasitic as well as two saprophytic Streptomyces species did not produce this enzyme when grown on either of the two potato media. This fact is clearly shown in Table Xll.

The maceration of the slices in the potato tissue medium was effected by two parasitic strains of S. scabies L27, and No. 10246 and by one saprophytic spp. of Streptomyces, S.122, after 6-days of growth. One parasitic and one saprophytic species L45 and S.402 respectively, did not produce the enzyme after 19-days growth. After this period the cultures were becoming dehydrated.

Sterile extracts from the above cultures were prepared by passing the culture liquid through a Seitz filter. Ten ml. of the extracts were placed in separate test tubes

TABLE X11

THE PRODUCTION OF PROTOPECTINASE BY STREPTOMYCES SPECIES WHILE GROWING ON SLICED POTATO TISSUE MEDIUM

| ISOLATE | | DEGREE OF MACERATION | NO. OF DAYS GROWTH |
|---|---|---|---|
| L27 | Virulent | + + + + | 6 |
| L45 | " | - | 19 |
| 10246 | " | + + + + | 9 |
| S.403 | Saprophytic | - | 19 |
| S.122 | " | + + + + | 6 |

+ + + + Complete Maceration - No Maceration

and slices of fresh potato tissue were added. The controls were prepared by adding the tissue to 10 ml. of boiled extract. The tubes were placed in a water bath maintained at 35°C. Medium extracts from the parasitic strain L27 and the saprophytic species S.122 effected complete maceration of the tissue after 18 hours. Medium extracts from the parasitic strain L45 and the saprophytic species S.403 did not show any protopectinase enzyme activity, even when extracts were prepared from the medium after 19-days growth. There was no maceration of the tissue in the boiled extracts after 18 hours.

When 10 ml. of a phosphate buffer at pH 6.5, were added to the potato tissue medium instead of water the results were very similar. The results of this experiment are presented in Table XIII.

In the case of the parasitic strain L27 the tissue was macerated by its culture extracts after 7-days growth. The culture extracts of two avirulent strains of S. scabies effected maceration after 13-days growth. Again the parasitic strain L45 failed to produce the enzyme after 19-days growth. Within the saprophytic group three species S.106, S.122 and S.129, produced the macerating enzyme after 7-days growth, while one other species, S.403 did not produce the tissue macerating enzyme even after 19-days growth.

TABLE X111

THE PRODUCTION OF PROTOPECTINASE BY STREPTOMYCES SPECIES WHILE GROWING ON FRESH POTATO TISSUE IN A PHOSPHATE BUFFER AT pH 6.5

| ISOLATE | | AGE OF CULTURE EXTRACT 5 DAYS | 7 DAYS | 13 DAYS | 19 DAYS |
|---|---|---|---|---|---|
| L27 | Virulent | + + | + + + + | | |
| L45 | " | - | - | - | - |
| S.429 | Avirulent | - | - | + + + + | |
| S.449 | " | - | - | + + + + | |
| S.106 | Saprophytic | + + | + + + + | | |
| S.122 | " | + | + + + + | | |
| S.129 | " | + | + + + + | | |
| S.403 | " | - | - | - | - |

+ + + + Complete maceration. - No maceration.

When the actinomycetes were grown on the potato decoction medium the results were similar to those obtained when these organisms were grown on fresh potato tissue medium. Medium extracts were made from the cultures after 3-days growth and every day thereafter up to 19 days. Standard size slices (0.5 mm. X 5.0 mm. X 20 mm.) of potato tissue were added to 10 ml. of the extracts and the degree of maceration was determined. Tests were conducted at 35°C. The effect of cell-free extracts of cultures on potato tuber tissue is presented in Table XlV.

TABLE XlV

THE PRODUCTION OF PROTOPECTINASE BY STREPTOMYCES SPECIES WHILE GROWING ON POTATO DECOCTION MEDIUM

| | | MACERATION OF POTATO TISSUE | AGE OF CULTURE EXTRACT |
|---|---|---|---|
| L27 | Virulent | + + + + (18 hr.) | 5 days |
| L45 | " | - " | 19 days |
| L38 | " | + + + + " | 6 days |
| S.122 | Saprophytic | + + + + " | 6 days |
| S.106 | " | + + + + " | 6 days |
| S.416 | " | - " | 19 days |

+ + + + Complete maceration. - No maceration.

Medium extracts from 5-day old cultures of two parasitic strains, L27 and L38, of S. scabies caused complete maceration of tissue in 18 hours. Extracts from 6-day old cultures of two saprophytic species S.122 and S.106 also effected maceration after 18 hours. Medium extracts from cultures of one parasitic strain L45 and one saprophytic species S.416 did not produce any maceration of tissue, even when the extracts were made after 19-days of growth.

When it was found that a number of the Streptomyces produced the enzyme protopectinase on potato decoction medium it was decided, to use this medium because of its relative ease of preparation, to obtain enzyme preparations to be used in a study of some of the properties of the enzyme. The parasitic strain of S. scabies L27 was used for producing the enzyme because it was the most active in this respect. Culture extracts were made after 7-days of growth. Crude preparations of the enzyme were obtained by the following method. Cell-free extracts were prepared by passing the culture liquid through a Seitz filter. The filtrate was adjusted to pH 6.5 with 0.1 N hydrochloric acid and the enzyme was precipitated out with 95% ethyl alcohol. The alcohol was added at the rate of four ml. for every one ml. of extract. This suspension was allowed to stand for two hours and the precipitate that formed was collected by centrafuging at 2000 r.p.m. and drying at 30°C. The precipitate obtained from 100 ml. of extract was

dissolved in 25 ml. of distilled water. This solution was filtered through a Watman No. 1 filter paper and the filtrate was used for the enzymatic tests.

The effect of the hydrogen-ion concentration and the effect of heat treatment on the protopectinase activity of the filtrate was determined.

Changes in hydrogen-ion concentration of the enzyme solution were made by adding 0.1 N hydrochloric acid or 0.1 N sodium hydroxide as required. Test tubes containing 10 ml. of the enzyme solution adjusted to pH levels of 5.6, 6.0, 7.0, 7.5 and 8.0 were placed in a water bath which was maintained at a temperature of 35°C. After the enzyme solutions had reached the proper temperature standard slices of potato tissue were added and the time required for tissue maceration was determined. The results are presented in Table XV.

The enzyme was most active when in a neutral or slightly alkaline solution, although it showed considerable activity over a wide pH range. More time was required for complete maceration of the tissue as the acidity of the solution was increased. There was also a slight inhibition of activity when the enzyme solution was at pH 8.0.

The effect of heat treatment on the protopectinase activity of the filtrates was determined in the following

TABLE XV

EFFECT OF HYDROGEN-ION CONCENTRATION ON PROTOPECTINASE ACTIVITY OF CULTURE FILTRATES

| pH OF ENZYME SOLUTION | TIME REQUIRED TO MACERATE POTATO TISSUE (35°C.) | | | |
|---|---|---|---|---|
| | 4 hr. | 5 hr. | 6 hr. | 7 hr. |
| 8.0 | + | + + + + | | |
| 7.5 | + + + + | | | |
| 7.0 | + + + + | | | |
| 6.0 | - | + | + + + + | |
| 5.6 | - | - | + | + + + + |

+ + + + Complete maceration. - No maceration.

manner: 10 ml. portions of the crude protopectinase enzyme solution, at pH 7.0, were heated for 10 minutes in a water bath maintained at the following temperature, 60°C, 70°C, 80°C, 90°C, and 100°C. After the required heat treatment the tubes were removed, cooled under running water, and placed in a water bath maintained at 35°C. When the temperature of the enzyme solution had reached equilibrium, standard slices of potato tissue were added and the time required for complete maceration of the tissue was determined. The control was prepared from an unheated enzyme solution. The results are presented in Table XVl.

TABLE XVl

THE MACERATION PRODUCED BY CULTURE FILTRATES OF POTATO TISSUE AFTER A 10 MINUTE HEAT TREATMENT ( OF THE ENZYME AT VARIOUS TEMPERATURES )

| TIME FOR COMPLETE MACERATION | HEAT TREATMENT FOR 10 MINUTES | | | | | |
|---|---|---|---|---|---|---|
| | CONTROL | 60°C. | 70°C. | 80°C. | 90°C. | 100°C. |
| 4 hr. | + + + + | - | - | - | - | - |
| 5 hr. | + + + + | + | - | - | - | - |
| 11 hr. | + + + + | + + | - | - | - | - |
| 18 hr. | + + + + | + + + + | + | - | - | - |

+ + + + Complete maceration. - No maceration.

Potato tissue in the unheated control was completely macerated in four hours. The enzyme solution which had been

heated to 60°C. for 10 minutes effected complete maceration of tissue in 18 hours, while the solution which had been heated to 70°C. for 10 minutes showed only a slight amount of maceration after 18 hours at 35°C. There was no maceration of tissue in the enzyme solutions which had been heated to 80°C., 90°C, and 100°C. after 18 hours.

## PRODUCTION OF OTHER PECTOLYTIC ENZYMES

The parasitic strains of Streptomyces scabies L27 and L45 and two saprophytic species S.122 and S.403 were grown on potato decoction medium and sliced potato tissue medium. Extracts were made from the media after 5, 7, 13 and 19 days of growth and determinations of the presence of the enzymes pectin-polygalacturonase and pectin-methylesterase were made.

Extracts from both media after 7 days growth of the parasitic strain L27, were treated with 95% ethyl alcohol and the resultant precipitate was collected, dried and redissolved in distilled water. This crude enzyme preparation was then tested for the presence of pectin-methylesterase and pectin-polygalacturonase.

The presence of pectin-polygalacturonase was determined by measuring the change of viscosity of a 1% pectic acid solution exposed to the preparation. According to Kertesz (1951) the measurement of viscosity changes is a very sensitive

indication of even traces of pectin-polygalacturonase. One gm. of pectic acid was dissolved in 100 ml. of distilled water at pH 4.6. The solution was filtered and made up to the required pH by the addition of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. Two point five ml. of this substrate were placed in a test tube and 0.5 ml. of the enzyme preparation was added. Viscosity measurements were made at the beginning of the experiment to determine the initial viscosity of the mixture. Appropriate controls with boiled enzyme solution were included in the tests which were conducted at 30°C.

No pectin-polygalacturonase was detected in any of the culture extracts or in the crude enzyme preparations even after 24 hr. incubation.

The presence of pectin-methylesterase was determined by a method described by Kertesz (1951). One ml. of the extract was added to 25 ml. of a slightly acid 1% pectin solution and the mixture was titrated with 0.1 N sodium hydroxide in the presence of methyl red indicator until the mixture lost its last pink tint from one drop of alkali. In the presence of active pectin-methylesterase, the mixture will turn red in less than 10 minutes. The tests were conducted at 30°C.

No pectin-methylesterase was detected in any of the culture extracts or in the crude enzyme preparations even after 24 hr. incubation.

## DISCUSSION

The respiration of potato tissue was depressed by media extracts from cultures of one parasitic strain No. 10246 of Streptomyces scabies. This reduced rate of respiration was effected with extracts from 3 to 11-day-old cultures grown on Czapek's agar. However, there was no effect on respiration with medium extracts from cultures after 12 days growth. When this strain was grown in shake cultures of Czapek's solution, similar reductions in respiration were effected with 5 to 12-day-old culture extracts, while extracts from older cultures effected an increase in potato tissue respiration. It appears that this parasitic strain of the organism produced a respiratory inhibitory substance which was subsequently destroyed by sustained growth of the organism. Whether the inhibitory substance was eventually utilized by the organism as a nutritive source or whether it was detoxified by some metabolic process remains to be ascertained.

The inhibitory substance was found to be thermolabile; its activity being destroyed at 60°C. for 10 minutes. It would appear from this that the inhibitory substance was proteinaceous in nature.

The predominant respiratory enzyme in the potato is polyphenoloxidase, a copper proteid, which has oxygen and polyphenols as its specific reaction partners. It is possible that

the inhibitory substance either inhibited the action of polyphenoloxidase or adversely affected some portion of the enzyme substrate.

The parasitic strain No. 10246 also produced a fluorescent compound when growing on both solid and liquid Czapek's medium. There was a partial correlation for a time, between the synthesis of this compound and that of the inhibitory substance, but eventually the inhibitory substance was destroyed while the fluorescent substance increased.

Medium extracts from two other parasitic cultures of S. scabies, designated as $P_1$ and $P_3$, stimulated the respiration of potato tissue. Medium extracts from another parasitic strain, L27, growing for 3 days on potato decoction medium, slightly reduced the respiration while extracts from 6-day-old cultures had no effect on the respiration rate of potato tissue. Extracts of culture L27, 8 and 10-days old, increased respiration of potato tissue.

There was a difference in the severity of scab produced by the strains of S. scabies but there did not appear to be any difference in the type of scab lesion produced. Also there was no correlation between severity of scab caused by these strains and the effect of their extracts on the respiration of potato tissue.

The fact that extracts from a mildly virulent strain of S. scabies depressed respiration of potato tissue while another more virulent strain increased it makes it difficult to draw any definite conclusions respecting the effect, if any, of altered host respiration upon the parasitism and pathogenicity of S. scabies. In this connection it is interesting to note that considerable evidence has now accumulated that the effect which a pathogen has on its host may have a bearing upon the reaction of the host to its presence. Farkas and Kiraly (1955) have reviewed the studies made by various workers and suggest that there appears to be a correlation existing between respiratory changes following infection by pathogens and resistance and susceptibility of the host. Bach (1912) suggested that the tissue respiration of the host may play a part in protecting plants against infection by oxidizing the toxins produced by the pathogens. Ruben and his co-workers (1953) and again in (1955) have found very suggestive evidence for the protective nature of oxidative processes from detailed studies carried out on host-parasite complexes such as potato - Phytophthora, cabbage - Botrytis, and citrus - Penicillium. The data indicates that the respiration of resistant varieties is generally more intensive than that of the susceptible ones and the increase in respiration, brought about by infection, is more pronounced in resistant plants. Fuchs and Kotte (1954) have demonstrated that resistance

of potato to Phytophthora depends on oxidative mechanisms. In the presence of respiratory inhibitors the resistant variety loses this property and becomes susceptible.

In contradiction to the above mentioned authors, Suchorukov (1952) concluded that the parasitically increased respiration of the host favors the development of the fungus. Sempio (1943) (1946) and again in (1950) studied the ratio of photosynthesis and respiration with regards to resistance of wheat to powdery mildew and reached the conclusion that high ratios (i.e. more anabolic type of metabolism) may be related to resistance.

A comparison of the main trends in the past with those prevailing in the more recent works reveals particularly remarkable changes in the basic concepts. It is generally believed that the classical approach to the elucidation of the nature of resistance, i.e. the simple comparison of resistant and susceptible plants from several physiological-chemical aspects, have yielded little information so far in regard to the basic question. The prepondence of observation indicates that the resistance results from an interaction between host and parasite, the protective mechanisms being activated exclusively and specifically by the pathogens.

Five strains of Streptomyces scabies and 4 saprophytic Streptomyces species produced the pectolytic enzyme

protopectinase when potato was the substrate. One parasitic strain and two saprophytic strains were consistant in not producing this enzyme. None of the cultures produced any detectable amounts of two other pectolytic enzymes, pectin-methylesterase and pectin-polygalacturonase.

It would appear from the data presented that Streptomyces species do not utilize the breakdown products of potato protopectin. If they did one would expect considerable pectin-methylesterase and pectin-polygalacturonase activity in the extracts from the potato media on which the organisms grew. However, in this case the presence of the methylesterase in not as important as the presence of the polygalacturonase. This is explained by the fact that as the Streptomyces species grow, the potato media become alkaline and under these conditions the breakdown products of protopectin (pectins) can be de-esterified. Then in order for the de-esterified compounds (polygalacturonic acid units) to be hydrolized to the utilizable mono unit of galacturonic acid, polygalacturonase is necessary.

It is possible that the parasitism of S. scabies is enhanced by its ability to produce enzymes that dissolve the middle lamella. However, since at least one parasitic strain cannot produce this enzyme on either of the two potato media and that saprophytic species can, would lead one to conclude

that protopectinase production is not prerequisite to the parasitism of S. scabies.

If protopectinase is produced in the potato tuber by S. scabies under natural growing conditions one would expect a soft-rot rather than a dry condition about the scab pustule. It is conceivable that only very small amounts of protopectinase are produced in the growing tuber and that its activity is short lived due to absorption or inactivation by the plant tissues. Wood (1955) states that possibly there are substances in the potato sap which inhibit, differentially, the activity of pectolytic enzymes.

The respiratory and pectolytic enzymes studied had no demonstrable role, at least by the methods employed, in determining the parasitism and pathogenicity of S. scabies.

## SUMMARY

(1) Studies were conducted to determine some of the physiological mechanisms involved in the parasitism of Streptomyces scabies. The first of these studies was concerned with the effect of medium extracts derived from cultures of the organism, on the respiration of potato tuber tissue. The second investigation was undertaken to determine if parasitic strains of S. scabies as well as saprophytic species of Streptomyces could produce pectolytic enzymes when these organisms were grown on potato tuber tissue media.

(2) A method of isolation of Actinomycetes with minimal contamination was devised and is described. This technique showed that isolations from soil,known to produce scabby tubers, and scabby potato tubers were facilitated by a 10 minute treatment of each in phenol at a dilution of 1: 140.

(3) A method of testing the pathogenicity of S. scabies utilizing detached axillary Green Mountain tubers was also devised and described.

(4) The effect of medium extracts from four parasisic strains of S. scabies and two saprophytic species of Streptomyces on the respiration of potato tuber tissue was studied. Extracts from the parasitic strain No. 10246 depressed respiration while those from the other parasitic and

saprophytic species usually increased the respiration of the tissue. Respiratory studies proved to be of no apparent value in gaining an understanding of the parasitism of S. scabies.

(5) The parasitic strain No. 10246 of S. scabies produced a fluorescent compound in both solid and liquid Czapek's medium. There was a partial correlation for a time, between the synthesis of this compound and that of the inhibitory substance, but eventually the inhibitory substance was destroyed while the fluorescent substance increased.

(6) A number of parasitic and saprophytic Streptomyces species produced the tissue-macerating enzyme, protopectinase. It was found that one parasitic as well as two saprophytic species did not produce this enzyme when cultured under identical conditions.

(7) The enzyme protopectinase from S. scabies No. 10246 had a thermal inactivation point of between 60°C. and 70°C. The enzyme was active over a wide pH range with its optimal activity between 7.0 and 7.5.

(8) No pectin-methylesterase and no pectin-polygalacturonase was produced by any of the Streptomyces species studied.

(9) The respiratory and pectolytic enzymes studied had no demonstrable role, at least by the methods employed, in determining the parasitism and pathogenicity of *S. scabies*.

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